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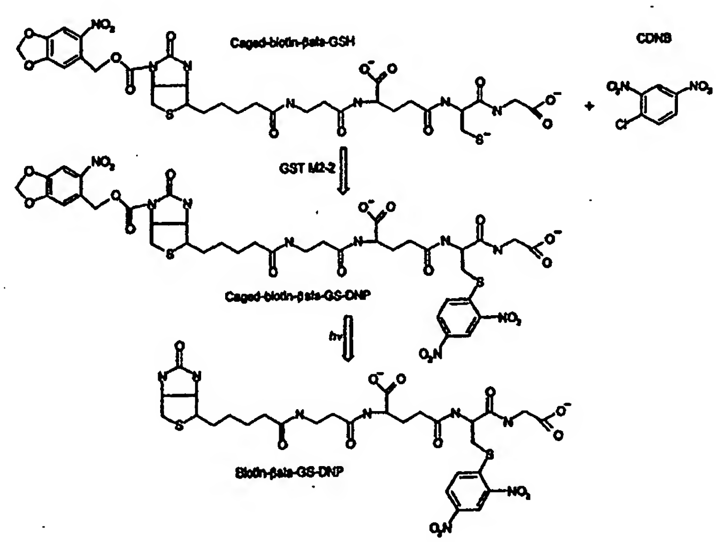

(57) Abstract

The invention describes a method for isolating one or more genetic elements encoding a gene product having a desired activity, comprising the steps of: (a) compartmentalising genetic elements into microcapsules; (b) expressing the genetic elements to produce their respective gene products within the microcapsules; (c) sorting the genetic elements which produce the gene product having the desired activity using a change in the optical properties of the genetic elements. The invention enables the *in vitro* evolution of nucleic acids and proteins by repeated mutagenesis and iterative applications of the method of the invention.

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OPTICAL SORTING METHOD

The present invention relates to methods for use in *in vitro* evolution of molecular libraries. In particular, the present invention relates to methods of selecting nucleic acids encoding gene products in which the nucleic acid and the activity of the encoded gene product are linked by compartmentation.

Evolution requires the generation of genetic diversity (diversity in nucleic acid) followed by the selection of those nucleic acids which result in beneficial characteristics. Because the nucleic acid and the activity of the encoded gene product of an organism are physically linked (the nucleic acids being confined within the cells which they encode) multiple rounds of mutation and selection can result in the progressive survival of organisms with increasing fitness. Systems for rapid evolution of nucleic acids or proteins *in vitro* advantageously mimic this process at the molecular level in that the nucleic acid and the activity of the encoded gene product are linked and the activity of the gene product is selectable.

Recent advances in molecular biology have allowed some molecules to be co-selected according to their properties along with the nucleic acids that encode them. The selected nucleic acids can subsequently be cloned for further analysis or use, or subjected to additional rounds of mutation and selection.

Common to these methods is the establishment of large libraries of nucleic acids. Molecules having the desired characteristics (activity) can be isolated through selection regimes that select for the desired activity of the encoded gene product, such as a desired biochemical or biological activity, for example binding activity.

Phage display technology has been highly successful as providing a vehicle that allows for the selection of a displayed protein by providing the essential link between nucleic acid and the activity of the encoded gene product (Smith, 1985; Bass et al. , 1990; McCafferty et al., 1990; for review see Clackson and Wells, 1994). Filamentous phage particles act as genetic display packages with proteins on the outside and the genetic elements which

encode them on the inside. The tight linkage between nucleic acid and the activity of the encoded gene product is a result of the assembly of the phage within bacteria. As individual bacteria are rarely multiply infected, in most cases all the phage produced from an individual bacterium will carry the same genetic element and display the same protein.

However, phage display relies upon the creation of nucleic acid libraries *in vivo* in bacteria. Thus, the practical limitation on library size allowed by phage display technology is of the order of 10^7 to 10^{11}, even taking advantage of λ phage vectors with excisable filamentous phage replicons. The technique has mainly been applied to selection of molecules with binding activity. A small number of proteins with catalytic activity have also been isolated using this technique, however, selection was not directly for the desired catalytic activity, but either for binding to a transition-state analogue (Widersten and Mannervik, 1995) or reaction with a suicide inhibitor (Soumillion et al., 1994; Janda et al., 1997). More recently there have been some examples of enzymes selected using phage-display by product formation (Atwell & Wells, 1999; Demartis *et al.*, 1999; Jestin *et al.*, 1999; Pederson, *et al.*, 1998), but in all these cases selection was not for multiple turnover.

Specific peptide ligands have been selected for binding to receptors by affinity selection using large libraries of peptides linked to the C terminus of the *lac* repressor *LacI* (Cull et al., 1992). When expressed in *E. coli* the repressor protein physically links the ligand to the encoding plasmid by binding to a lac operator sequence on the plasmid.

An entirely *in vitro* polysome display system has also been reported (Mattheakis et al., 1994; Hanes and Pluckthun, 1997) in which nascent peptides are physically attached *via* the ribosome to the RNA which encodes them. An alternative, entirely *in vitro* system for linking genotype to phenotype by making RNA-peptide fusions (Roberts and Szostak, 1997; Nemoto et al., 1997) has also been described.

However, the scope of the above systems is limited to the selection of proteins and furthermore does not allow direct selection for activities other than binding, for example catalytic or regulatory activity.

In vitro RNA selection and evolution (Ellington and Szostak, 1990), sometimes referred to as SELEX (systematic evolution of ligands by exponential enrichment) (Tuerk and Gold, 1990) allows for selection for both binding and chemical activity, but only for nucleic acids. When selection is for binding, a pool of nucleic acids is incubated with immobilised substrate. Non-binders are washed away, then the binders are released, amplified and the whole process is repeated in iterative steps to enrich for better binding sequences. This method can also be adapted to allow isolation of catalytic RNA and DNA (Green and Szostak, 1992; for reviews see Chapman and Szostak, 1994; Joyce, 1994; Gold et al., 1995; Moore, 1995).

However, selection for "catalytic" or binding activity using SELEX is only possible because the same molecule performs the dual role of carrying the genetic information and being the catalyst or binding molecule (aptamer). When selection is for "auto-catalysis" the same molecule must also perform the third role of being a substrate. Since the genetic element must play the role of both the substrate and the catalyst, selection is only possible for single turnover events. Because the "catalyst" is in this process itself modified, it is by definition not a true catalyst. Additionally, proteins may not be selected using the SELEX procedure. The range of catalysts, substrates and reactions which can be selected is therefore severely limited.

Those of the above methods that allow for iterative rounds of mutation and selection are mimicking *in vitro* mechanisms usually ascribed to the process of evolution: iterative variation, progressive selection for a desired the activity and replication. However, none of the methods so far developed have provided molecules of comparable diversity and functional efficacy to those that are found naturally. Additionally, there are no man-made "evolution" systems which can evolve both nucleic acids and proteins to effect the full range of biochemical and biological activities (for example, binding, catalytic and regulatory activities) and that can combine several processes leading to a desired product or activity.

There is thus a great need for an *in vitro* system that overcomes the limitations discussed above.

In Tawfik and Griffiths (1998), and in International patent application PCT/GB98/01889, we describe a system for *in vitro* evolution that overcomes many of the limitations described above by using compartmentalisation in microcapsules to link genotype and phenotype at the molecular level.

In Tawfik and Griffiths (1998), and in several embodiments of International patent application PCT/GB98/01889, the desired activity of a gene product results in a modification of the genetic element which encoded it (and is present in the same microcapsule). The modified genetic element can then be selected in a subsequent step.

Here we describe a further invention in which the modification of the genetic element causes a change in the optical properties of the element itself, and which has many advantages over the methods described previously.

BRIEF DESCRIPTION OF THE INVENTION

According to a first aspect of the present invention, there is provided a method for isolating one or more genetic elements encoding a gene product having a desired activity the expression of which may result, directly or indirectly, in the modification of an optical property of a genetic element encoding the gene product, comprising the steps of:

(a) compartmentalising genetic elements into microcapsules;

(b) expressing the genetic elements to produce their respective gene products within the microcapsules;

(c) sorting the genetic elements which produce the gene product(s) having the desired activity according to the changed optical properties of the genetic elements.

The microcapsules according to the present invention compartmentalise genetic elements and gene products such that they remain physically linked together.

As used herein, a genetic element is a molecule or molecular construct comprising a nucleic acid. The genetic elements of the present invention may comprise any nucleic acid (for example, DNA, RNA or any analogue, natural or artificial, thereof). The nucleic acid component of the genetic element may moreover be linked, covalently or non-covalently, to one or more molecules or structures, including proteins, chemical entities and groups, and solid-phase supports such as beads (including nonmagnetic, magnetic and paramagnetic beads), and the like. In the method of the invention, these structures or molecules can be designed to assist in the sorting and/or isolation of the genetic element encoding a gene product with the desired activity.

Expression, as used herein, is used in its broadest meaning, to signify that a nucleic acid contained in the genetic element is converted into its gene product. Thus, where the nucleic acid is DNA, expression refers to the transcription of the DNA into RNA; where this RNA codes for protein, expression may also refer to the translation of the RNA into protein. Where the nucleic acid is RNA, expression may refer to the replication of this RNA into further RNA copies, the reverse transcription of the RNA into DNA and optionally the transcription of this DNA into further RNA molecule(s), as well as optionally the translation of any of the RNA species produced into protein. Preferably, therefore, expression is performed by one or more processes selected from the group consisting of transcription, reverse transcription, replication and translation.

Expression of the genetic element may thus be directed into either DNA, RNA or protein, or a nucleic acid or protein containing unnatural bases or amino acids (the gene product) within the microcapsule of the invention, so that the gene product is confined within the same microcapsule as the genetic element.

The genetic element and the gene product thereby encoded are linked by confining each genetic element and the respective gene product encoded by the genetic element within the same microcapsule. In this way the gene product in one microcapsule cannot cause a change in any other microcapsules. In addition, further linking means may be employed to link gene products to the genetic elements encoding them, as set forth below.

The term "microcapsule" is used herein in accordance with the meaning normally assigned thereto in the art and further described hereinbelow. In essence, however, a microcapsule is an artificial compartment whose delimiting borders restrict the exchange of the components of the molecular mechanisms described herein which allow the sorting of the genetic elements according to the function of the gene products which they encode.

Preferably, the microcapsules used in the method of the present invention will be capable of being produced in very large numbers, and thereby to compartmentalise a library of genetic elements which encodes a repertoire of gene products.

As used herein, a change in optical properties of the genetic elements refers to any change in absorption or emission of electromagnetic radiation, including changes in absorbance, luminescence, phosphorescence or fluorescence. All such properties are included in the term "optical". Genetic elements can be sorted, for example, by luminescence, fluorescence or phosphorescence activated sorting. In a preferred embodiment, flow cytometry is employed to sort genetic elements, for example, light scattering (Kerker, 1983) and fluorescence polarisation (Rolland et al., 1985) can be used to trigger flow sorting. In a highly preferred embodiment genetic elements are sorted using a fluorescence activated cell sorter (FACS) sorter (Norman, 1980; Mackenzie and Pinder, 1986).

Changes in optical properties may be direct or indirect. Thus, the change may result in the alteration of an optical property in the genetic element itself, or may lead indirectly to such a change. For example, modification of a genetic element may alter its ability to bind an optically active ligand, thus indirectly altering its optical properties.

Alternatively, imaging techniques can be used to screen thin films of genetic elements to allow enrichment for a genetic element with desirable properties, for example by physical isolation of the region where a genetic element with desirable properties is situated, or ablation of non-desired genetic elements. The genetic elements can be detected by luminescence, phosphorescence or fluorescence.

According to a preferred embodiment of the first aspect of the present invention, the sorting of genetic elements may be performed in one of essentially two techniques.

(I) In a first embodiment, the genetic elements are sorted following pooling of the microcapsules into one or more common compartments. In this embodiment, a gene product having the desired activity modifies the genetic element which encoded it (and which resides in the same microcapsule) so as to make it selectable as a result of its modified optical properties in a subsequent step. The reactions are stopped and the microcapsules are then broken so that all the contents of the individual microcapsules are pooled. The modification of the genetic element in the microcapsule may result directly in the modification of the optical properties of the genetic element. Alternatively, the modification may allow the genetic elements to be further modified outside the microcapsules so as to induce a change in their optical properties. Selection for the genetic elements with modified optical properties enables enrichment of the genetic elements encoding the gene product(s) having the desired activity. Accordingly, the invention provides a method according to the first aspect of the invention, wherein in step (b) the gene product having the desired activity modifies the genetic element encoding it to enable the isolation of the genetic element as a result in a change in the optical properties of the genetic element. It is to be understood, of course, that modification may be direct, in that it is caused by the direct action of the gene product on the genetic element, or indirect, in which a series of reactions, one or more of which involve the gene product having the desired activity, leads to modification of the genetic element.

(II) In a second embodiment, the genetic elements may be sorted by a multi-step procedure, which involves at least two steps, for example, in order to allow the exposure of the genetic elements to conditions which permit at least two separate reactions to occur. As will be apparent to persons skilled in the art, the first microencapsulation step of the invention advantageously results in conditions which permit the expression of the genetic elements - be it transcription, transcription and/or translation, replication or the like. Under these conditions, it may not be possible to select for a particular gene product activity, for example because the gene product may not be active under these conditions, or because the expression system contains an interfering activity. The invention therefore

provides a method according to the first aspect of the present invention. wherein step (b) comprises expressing the genetic elements to produce their respective gene products within the microcapsules, linking the gene products to the genetic elements encoding them and isolating the complexes thereby formed. This allows for the genetic elements and their associated gene products to be isolated from the capsules before sorting according to gene product activity takes place. In a preferred embodiment, the complexes are subjected to a further compartmentalisation step prior to isolating the genetic elements encoding a gene product having the desired activity. This further compartmentalisation step, which advantageously takes place in microcapsules, permits the performance of further reactions, under different conditions, in an environment where the genetic elements and their respective gene products are physically linked. Eventual sorting of genetic elements may be performed according to embodiment (I) above.

The "secondary encapsulation" may also be performed with genetic elements linked to gene products by other means, such as by phage display, polysome display, RNA-peptide fusion or lac repressor peptide fusion.

The selected genetic element(s) may also be subjected to subsequent, optionally more stringent rounds of sorting in iteratively repeated steps, reapplying the method of the invention either in its entirety or in selected steps only. By tailoring the conditions appropriately, genetic elements encoding gene products having a better optimised activity may be isolated after each round of selection.

Additionally, the genetic elements isolated after a first round of sorting may be subjected to mutagenesis before repeating the sorting by iterative repetition of the steps of the method of the invention as set out above. After each round of mutagenesis, some genetic elements will have been modified in such a way that the activity of the gene products is enhanced.

Moreover, the selected genetic elements can be cloned into an expression vector to allow further characterisation of the genetic elements and their products.

In a second aspect, the invention provides a product when selected according to the first aspect of the invention. As used in this context, a "product" may refer to a gene product, selectable according to the invention, or the genetic element (or genetic information comprised therein).

In a third aspect, the invention provides a method for preparing a gene product, the expression of which may result, directly or indirectly, in the modification the optical properties of a genetic element encoding it, comprising the steps of:

(a) preparing a genetic element encoding the gene product;
(b) compartmentalising genetic elements into microcapsules;
(c) expressing the genetic elements to produce their respective gene products within the microcapsules;
(d) sorting the genetic elements which produce the gene product(s) having the desired activity using the changed optical properties of the genetic elements; and
(e) expressing the gene product having the desired activity .

In accordance with the third aspect, step (a) preferably comprises preparing a repertoire of genetic elements, wherein each genetic element encodes a potentially differing gene product. Repertoires may be generated by conventional techniques, such as those employed for the generation of libraries intended for selection by methods such as phage display. Gene products having the desired activity may be selected from the repertoire, according to the present invention, according to their ability to modify the optical properties of the genetic elements in a manner which differs from that of other gene products. For example, desired gene products may modify the optical properties to a greater extent than other gene products, or to a lesser extent, including not at all.

In a fourth aspect, the invention provides a method for screening a compound or compounds capable of modulation the activity of a gene product, the expression of which may result, directly or indirectly, in the modification of the optical properties of a genetic element encoding it, comprising the steps of:

(a) preparing a repertoire of genetic elements encoding gene product;
(b) compartmentalising genetic elements into microcapsules;

(c) expressing the genetic elements to produce their respective gene products within the microcapsules;

(d) sorting the genetic elements which produce the gene product(s) having the desired activity using the changed optical properties of the genetic elements; and

(e) contacting a gene product having the desired activity with the compound or compounds and monitoring the modulation of an activity of the gene product by the compound or compounds.

Advantageously, the method further comprises the step of:

(g) identifying the compound or compounds capable of modulating the activity of the gene product and synthesising said compound or compounds.

This selection system can be configured to select for RNA, DNA or protein molecules with catalytic, regulatory or binding activity.

Brief Description of the Figures

Figure 1. *Dihydrofolate reductase can be expressed from genes in vitro translated in solution and genes attached to paramagnetic beads with identical efficiency.* The DHFR activity resulting from *in vitro* translation of *fol*A genes in solution or *fol*A genes attached to paramagnetic microbeads is determined by monitoring the oxidation of NADPH to NADP spectrophotometrically at 340 nm and activity is calculated by initial velocities under So>>K_M conditions (υmax). (◆), translated from genes in solution; (■), translated from genes attached to microbeads.2.

Figure 2. *Epifluorescence microscopy of water-in-oil emulsions* demonstrating that GFP can be translated in vitro from genes attached to single microbeads encapsulated in the aqueous compartments of the emulsions and the translated gene-product bound back the microbeads making them fluorescent.

Figure 3. *Flow cytometric analysis of GFP expression in microcapsules and in situ binding to the genetic element (microbeads).* A: The light scattering characteristics of the

beads before reaction. 75% of beads run as single beads. B: The light scattering characteristics of the beads after in vitro translation reaction. About 50% of beads fall into the gate for single beads. C: Fluorescence from microbeads (gated for single beads only) coated with T7-GFP gene and anti-GFP polyclonal antibody is significantly higher than the signal from the beads where either the GFP gene or the anti-GFP antibody were omitted.

Figure 4. *Synthesis of Biotin-GS-DNP by the human glutathione S-transferase M2-2 (GST M2-2) catalysed reaction of 1-chloro-2,4-dinitrobenzene (CDNB; Sigma) with reduced biotinylated-glutathione (Biotin-GSH).*

Figure 5. *Detecting paramagnetic beads coated with the product of an enzyme catalysed reaction by flow cytometry.* Sera-Mag™ streptavidin-coated magnetic microparticles incubated with Biotin-GS-DNP made by the GST M2-2 catalysed reaction of Biotin-GSH and CDNB. The captured Biotin-GS-DNP was detected by incubation of the microparticles with a mouse anti-dinitrophenol antibody followed by a (FITC)-conjugated $F(ab')_2$ fragment goat anti-mouse IgG, F(ab')2 fragment. After washing, 2 x 10^5 microparticles were analysed by flow cytometry. All reagents, no reagents omitted from the enzymatic synthesis of with Biotin-GS-DNP; minus GST, the enzyme GST M2-2 was omitted from the synthesis; minus biotin-GSH, biotin-GSH was omitted from the synthesis; minus CDNB, CDNB was omitted from the synthesis.

Figure 6. *Synthesis of MeNPO-CO-Biotin-β-Ala-GSH (caged-biotin-βala-GSH).* Acetyl chloride (5 ml) was added to anhydrous methanol (80 ml). The stirred solution was allowed to cool down and d-biotin (4 g) was added. After over-night stirring the solvents were evaporated in vacuum to afford a white solid. The solid was triturated with ether, filtered and dried under vacuum (in the presence of phosphorus pentoxide) and stored at –20°C.

Figure 7. *Reaction of caged-biotin-βala-GSH with 1-chloro-2,4-dinitrobenzene (CDNB) and photochemical uncaging of the biotin group.*

Figure 8. *Reaction of caged-biotin-βala-GSH with 4-chloro-3-nitrobenzoate (CNB) and photochemical uncaging of the biotin group*

Figure 9. *Human GST M2-2 catalyses the reaction of caged-biotin-βala-GSH with CDNB and CNB in solution and the reaction products can be uncaged by UV irradiation, captured on beads and detected using fluorescently labelled anti-product antibodies and flow cytometry.*

Panel A: light scattering characteristics of beads and gate for single beads (R1). Panel B: fluorescence from microbeads (gated through R1) from reactions with CDNB. Panel C: fluorescence from microbeads (gated through R1) from reactions with CNB. Signals from microbeads from reactions with and without GST M2-2 are annotated +enz and –enz respectively. Signals from microbeads from reactions which were UV irradiated and those which were not are annotated +UV and –UV respectively.

Figure 10. *Flow cytometry can be used to distinguish beads from aqueous compartments of an emulsion containing GST M2-2 from beads from compartments without GST M2-2 by using caged-biotinylated-βAla-GSH and CNB as substrates.*

Panel A: light scattering characteristics of a mixture of a mixture of 1.0 μm diameter nonfluorescent neutravidin labelled microspheres (Molecular Probes, F-8777) or 0.93 μm diameter streptavidin-coated polystyrene beads (Bangs Laboratories) and gates set for single Bangs beads (R1) and single Molecular Probes beads (R2). Panel B: fluorescence from microbeads taken from a non-emulsified mixture of 98% Bangs beads (without GST) and 2% Molecular Probes beads (with GST). Panel C: fluorescence from microbeads taken from a mixture of two emulsions in a ratio of 98% emulsion containing Bangs beads (without GST) and an emulsion containing 2% Molecular Probes beads (with GST). Panel D: fluorescence from microbeads taken from a non-emulsified mixture of 98% Molecular Probes beads (without GST) and 2% Bangs beads (with GST). Panel E: fluorescence from microbeads taken from a mixture of two emulsions in a ratio of 98% emulsion containing Molecular Probes beads (without GST) and an emulsion containing 2% Bangs beads (with GST). Fluorescence of ungated beads (No gate), beads gated through R1 (R1) and beads gated through R2 (R2) are overlayed.

Figure 11. *Human GST M2-2 transcribed and translated in vitro in the aqueous compartments of a water-in oil emulsion catalyses a reaction which gives rise to a change in the fluorescence properties of co-compartmentalised microspheres.*

Panel A: light scattering characteristics of beads and gate for single beads (R1). Panel B: fluorescence from microbeads (gated through R1) from non-emulsified reactions. Panel C: fluorescence from microbeads (gated through R1) emulsified reactions. Signals from microbeads from reactions with and without GSTM2-2.LMB2-3 DNA are annotated +DNA and –DNA respectively. Signals from microbeads from reactions with and without recombinant GST M2-2 are annotated +GST and –GST respectively.

Figure 12. *Synthesis of the caged-biotinylated substrate EtNP-BzGlu-cagedBiotin (17).*

Figure 13. *Hydrolysis of the PTE substrate EtNP-Bz-Glu-cagedBiotin (17) to yield the product Et-Bz-Glu-cagedBiotin, and uncaging of both substrate and product to yield the corresponding biotinylated substrate (EtNP-Bz-Glu-Biotin) and product (EtNP-Bz-Glu-Biotin)*

Figure 14. *Preparation of protein conjugates of a PTE substrate and product for immunisation and ELISA.*

Figure 15. *PTE catalyses the reaction of EtNP-Bz-Glu-cagedBiotin in the presence of streptavidin-coated beads, and the reaction products uncaged by UV irradiation, are captured on beads and detected using fluorescently labelled anti-product antibodies and flow cytometry.*

Panel A: light scattering characteristics of the beads and gate selected for single beads (R2). Panel B: fluorescence from beads (gated through R2) from reactions with 10 µM EtNP-Bz-Glu-cagedBiotin in the presence of *in vitro* translated OPD.LMB3-2biotin DNA fragments (OPD) or M.Hae*III*.LMB3-2biotin DNA fragments (M.*Hae*III). Panel C: As B

but with 20 μM EtNP-Bz-Glu-cagedBiotin. Panel D: As B but with 50 μM EtNP-Bz-Glu-cagedBiotin.

Figure 16. *Reaction of EtNP-Bz-Glu-cagedBiotin in the presence of beads to which genetic elements encoding the phosphotriesterase tagged with the Flag peptide (N-Flag-OPD.LMB3-2biotin) or another enzyme (N-Flag-M.HaeIII.LMB3-2biotin) were attached alongside with an antibody that binds the Flag peptide. The beads were reacted and subsequently analysed by flow-cytometry as described in the text.*
Panel A: light scattering characteristics of beads and gate for single beads (R1). Panel B: fluorescence from microbeads (gated through R1) to which were attached N-Flag-OPD.LMB3-2biotin DNA fragments (OPD) or M.Hae*III*.LMB3-2biotin DNA fragments (M.*Hae*III) from reactions with 12.5 μM EtNP-Bz-Glu-cagedBiotin. Panel C: As B but with 25 μM EtNP-Bz-Glu-caged-Biotin.

Figure 17. *E. coli BirA transcribed and translated in vitro catalyses a reaction which gives rise to a change in the fluorescence properties of substrate-labelled microspheres in the aqueous compartments of a water-in oil emulsion and in bulk solution.*

Figure 18. *Flow cytometric analysis of samples prepared for the sorting experiment.*

Figure 19. *Fluorescence-activated flow cytometric sorting of the genetic elements.*
Panel A: Samples #1 to #4 before sorting and after sorting. Panel B: Genes recovered from individual beads sorted from sample #3 sorted into a 96-well plate. Panel C: Genes recovered from individual beads sorted from sample #4 sorted into a 96-well plate. DNA markers (M) are ϕX174-HaeIII digest.

(A) GENERAL DESCRIPTION

The microcapsules of the present invention require appropriate physical properties to allow the working of the invention.

First, to ensure that the genetic elements and gene products may not diffuse between microcapsules, the contents of each microcapsule are preferably isolated from the contents of the surrounding microcapsules, so that there is no or little exchange of the genetic elements and gene products between the microcapsules over the timescale of the experiment.

Second, the method of the present invention requires that there are only a limited number of genetic elements per microcapsule. This ensures that the gene product of an individual genetic element will be isolated from other genetic elements. Thus, coupling between genetic element and gene product will be highly specific. The enrichment factor is greatest with on average one or fewer genetic elements per microcapsule, the linkage between nucleic acid and the activity of the encoded gene product being as tight as is possible, since the gene product of an individual genetic element will be isolated from the products of all other genetic elements. However, even if the theoretically optimal situation of, on average, a single genetic element or less per microcapsule is not used, a ratio of 5, 10, 50, 100 or 1000 or more genetic elements per microcapsule may prove beneficial in sorting a large library. Subsequent rounds of sorting, including renewed encapsulation with differing genetic element distribution, will permit more stringent sorting of the genetic elements. Preferably, there is a single genetic element, or fewer, per microcapsule.

Third, the formation and the composition of the microcapsules advantageously does not abolish the function of the machinery the expression of the genetic elements and the activity of the gene products.

The appropriate system(s) may vary depending on the precise nature of the requirements in each application of the invention, as will be apparent to the skilled person.

A wide variety of microencapsulation procedures are available (see Benita, 1996) and may be used to create the microcapsules used in accordance with the present invention.

Indeed, more than 200 microencapsulation methods have been identified in the literature (Finch, 1993).

These include membrane enveloped aqueous vesicles such as lipid vesicles (liposomes) (New, 1990) and non-ionic surfactant vesicles (van Hal et al., 1996). These are closed-membranous capsules of single or multiple bilayers of non-covalently assembled molecules, with each bilayer separated from its neighbour by an aqueous compartment. In the case of liposomes the membrane is composed of lipid molecules; these are usually phospholipids but sterols such as cholesterol may also be incorporated into the membranes (New, 1990). A variety of enzyme-catalysed biochemical reactions, including RNA and DNA polymerisation, can be performed within liposomes (Chakrabarti et al., 1994; Oberholzer et al., 1995a; Oberholzer et al., 1995b; Walde et al., 1994; Wick & Luisi, 1996).

With a membrane-enveloped vesicle system much of the aqueous phase is outside the vesicles and is therefore non-compartmentalised. This continuous, aqueous phase is removed or the biological systems in it inhibited or destroyed (for example, by digestion of nucleic acids with DNase or RNase) in order that the reactions are limited to the microcapsules (Luisi et al., 1987).

Enzyme-catalysed biochemical reactions have also been demonstrated in microcapsules generated by a variety of other methods. Many enzymes are active in reverse micellar solutions (Bru & Walde, 1991; Bru & Walde, 1993; Creagh et al., 1993; Haber et al., 1993; Kumar et al., 1989; Luisi & B., 1987; Mao & Walde, 1991; Mao et al., 1992; Perez et al., 1992; Walde et al., 1994; Walde et al., 1993; Walde et al., 1988) such as the AOT-isooctane-water system (Menger & Yamada, 1979).

Microcapsules can also be generated by interfacial polymerisation and interfacial complexation (Whateley, 1996). Microcapsules of this sort can have rigid, nonpermeable membranes, or semipermeable membranes. Semipermeable microcapsules bordered by cellulose nitrate membranes, polyamide membranes and lipid-polyamide membranes can all support biochemical reactions, including multienzyme systems (Chang, 1987; Chang,

1992; Lim, 1984). Alginate/polylysine microcapsules (Lim & Sun, 1980), which can be formed under very mild conditions, have also proven to be very biocompatible, providing, for example, an effective method of encapsulating living cells and tissues (Chang, 1992; Sun et al., 1992).

Non-membranous microencapsulation systems based on phase partitioning of an aqueous environment in a colloidal system, such as an emulsion, may also be used.

Preferably, the microcapsules of the present invention are formed from emulsions; heterogeneous systems of two immiscible liquid phases with one of the phases dispersed in the other as droplets of microscopic or colloidal size (Becher, 1957; Sherman, 1968; Lissant, 1974; Lissant, 1984).

Emulsions may be produced from any suitable combination of immiscible liquids. Preferably the emulsion of the present invention has water (containing the biochemical components) as the phase present in the form of finely divided droplets (the disperse, internal or discontinuous phase) and a hydrophobic, immiscible liquid (an oil') as the matrix in which these droplets are suspended (the nondisperse, continuous or external phase). Such emulsions are termed "water-in-oil" (W/O). This has the advantage that the entire aqueous phase containing the biochemical components is compartmentalised in discreet droplets (the internal phase). The external phase, being a hydrophobic oil, generally contains none of the biochemical components and hence is inert.

The emulsion may be stabilised by addition of one or more surface-active agents (surfactants). These surfactants are termed emulsifying agents and act at the water/oil interface to prevent (or at least delay) separation of the phases. Many oils and many emulsifiers can be used for the generation of water-in-oil emulsions; a recent compilation listed over 16,000 surfactants, many of which are used as emulsifying agents (Ash and Ash, 1993). Suitable oils include light white mineral oil and non-ionic surfactants (Schick, 1966) such as sorbitan monooleate (Span™80; ICI) and polyoxyethylenesorbitan monooleate (Tween™80; ICI).

The use of anionic surfactants may also be beneficial. Suitable surfactants include sodium cholate and sodium taurocholate. Particularly preferred is sodium deoxycholate, preferably at a concentration of 0.5% w/v, or below. Inclusion of such surfactants can in some cases increase the expression of the genetic elements and/or the activity of the gene products. Addition of some anionic surfactants to a non-emulsified reaction mixture completely abolishes translation. During emulsification, however, the surfactant is transferred from the aqueous phase into the interface and activity is restored. Addition of an anionic surfactant to the mixtures to be emulsified ensures that reactions proceed only after compartmentalisation.

Creation of an emulsion generally requires the application of mechanical energy to force the phases together. There are a variety of ways of doing this which utilise a variety of mechanical devices, including stirrers (such as magnetic stir-bars, propeller and turbine stirrers, paddle devices and whisks), homogenisers (including rotor-stator homogenisers, high-pressure valve homogenisers and jet homogenisers), colloid mills, ultrasound and 'membrane emulsification' devices (Becher, 1957; Dickinson, 1994).

Aqueous microcapsules formed in water-in-oil emulsions are generally stable with little if any exchange of genetic elements or gene products between microcapsules. Additionally, we have demonstrated that several biochemical reactions proceed in emulsion microcapsules. Moreover, complicated biochemical processes, notably gene transcription and translation are also active in emulsion microcapsules. The technology exists to create emulsions with volumes all the way up to industrial scales of thousands of litres (Becher, 1957; Sherman, 1968; Lissant, 1974; Lissant, 1984).

The preferred microcapsule size will vary depending upon the precise requirements of any individual selection process that is to be performed according to the present invention. In all cases, there will be an optimal balance between gene library size, the required enrichment and the required concentration of components in the individual microcapsules to achieve efficient expression and reactivity of the gene products.

The processes of expression occurs within each individual microcapsule provided by the present invention. Both *in vitro* transcription and coupled transcription-translation become less efficient at sub-nanomolar DNA concentrations. Because of the requirement for only a limited number of DNA molecules to be present in each microcapsule, this therefore sets a practical upper limit on the possible microcapsule size. Preferably, the mean volume of the microcapsules is less that 5.2×10^{-16} m^3, (corresponding to a spherical microcapsule of diameter less than 10μm, more preferably less than 6.5×10^{-17} m^3 (5μm diameter), more preferably about 4.2×10^{-18} m^3 (2μm diameter) and ideally about 9×10^{-18} m^3 (2.6μm diameter).

The effective DNA or RNA concentration in the microcapsules may be artificially increased by various methods that will be well-known to those versed in the art. These include, for example, the addition of volume excluding chemicals such as polyethylene glycols (PEG) and a variety of gene amplification techniques, including transcription using RNA polymerases including those from bacteria such as *E. coli* (Roberts, 1969; Blattner and Dahlberg, 1972; Roberts et al., 1975; Rosenberg et al. , 1975) , eukaryotes e. g. (Weil et al. , 1979; Manley et al., 1983) and bacteriophage such as T7, T3 and SP6 (Melton et al., 1984); the polymerase chain reaction (PCR) (Saiki et al., 1988); Qb replicase amplification (Miele et al., 1983; Cahill et al., 1991; Chetverin and Spirin, 1995; Katanaev et al., 1995); the ligase chain reaction (LCR) (Landegren et al., 1988; Barany, 1991); and self-sustained sequence replication system (Fahy et al., 1991) and strand displacement amplification (Walker et al., 1992). Gene amplification techniques requiring thermal cycling such as PCR and LCR may be used if the emulsions and the *in vitro* transcription or coupled transcription-translation systems are thermostable (for example, the coupled transcription-translation systems can be made from a thermostable organism such as Thermus aquaticus).

Increasing the effective local nucleic acid concentration enables larger microcapsules to be used effectively. This allows a preferred practical upper limit to the microcapsule volume of about $5.2 \times 10^{-16}$$m^3$ (corresponding to a sphere of diameter 10μm).

The microcapsule size is preferably sufficiently large to accommodate all of the required components of the biochemical reactions that are needed to occur within the microcapsule. For example, *in vitro*, both transcription reactions and coupled transcription-translation reactions require a total nucleoside triphosphate concentration of about 2mM.

For example, in order to transcribe a gene to a single short RNA molecule of 500 bases in length, this would require a minimum of 500 molecules of nucleoside triphosphate per microcapsule (8.33×10^{-22} moles). In order to constitute a 2mM solution, this number of molecules is contained within a microcapsule of volume 4.17×10^{-19} litres (4.17×10^{-22} m^3 which if spherical would have a diameter of 93nm.

Furthermore, particularly in the case of reactions involving translation, it is to be noted that the ribosomes necessary for the translation to occur are themselves approximately 20nm in diameter. Hence, the preferred lower limit for microcapsules is a diameter of approximately 0.1µm (100nm).

Therefore, the microcapsule volume is preferably of the order of between 5.2×10^{-22} m^3 and 5.2×10^{-16} m^3 corresponding to a sphere of diameter between 0.1µm and 10µm, more preferably of between about 5.2×10^{-19} m3 and 6.5×10^{-17} m^3 (1µm and 5µm). Sphere diameters of about 2.6µm are most advantageous.

It is no coincidence that the preferred dimensions of the compartments (droplets of 2.6µm mean diameter) closely resemble those of bacteria, for example, *Escherichia* are 1.1-1.5 x 2.0-6.0 µm rods and *Azotobacter* are 1.5-2.0 µm diameter ovoid cells. In its simplest form, Darwinian evolution is based on a 'one genotype one phenotype' mechanism. The concentration of a single compartmentalised gene, or genome, drops from 0.4 nM in a compartment of 2 µm diameter, to 25 pM in a compartment of 5 µm diameter. The prokaryotic transcription/translation machinery has evolved to operate in compartments of ~1-2 µm diameter, where single genes are at approximately nanomolar concentrations. A single gene, in a compartment of 2.6 µm diameter is at a concentration of 0.2 nM. This gene concentration is high enough for efficient translation. Compartmentalisation in such

a volume also ensures that even if only a single molecule of the gene product is formed it is present at about 0.2 nM, which is important if the gene product is to have a modifying activity of the genetic element itself. The volume of the microcapsule is thus selected bearing in mind not only the requirements for transcription and translation of the genetic element, but also the modifying activity required of the gene product in the method of the invention.

The size of emulsion microcapsules may be varied simply by tailoring the emulsion conditions used to form the emulsion according to requirements of the selection system. The larger the microcapsule size, the larger is the volume that will be required to encapsulate a given genetic element library, since the ultimately limiting factor will be the size of the microcapsule and thus the number of microcapsules possible per unit volume.

The size of the microcapsules is selected not only having regard to the requirements of the transcription/translation system, but also those of the selection system employed for the genetic element. Thus, the components of the selection system, such as a chemical modification system, may require reaction volumes and/or reagent concentrations which are not optimal for transcription/translation. As set forth herein, such requirements may be accommodated by a secondary re-encapsulation step; moreover, they may be accommodated by selecting the microcapsule size in order to maximise transcription/translation and selection as a whole. Empirical determination of optimal microcapsule volume and reagent concentration, for example as set forth herein, is preferred.

A "genetic element" in accordance with the present invention is as described above. Preferably, a genetic element is a molecule or construct selected from the group consisting of a DNA molecule, an RNA molecule, a partially or wholly artificial nucleic acid molecule consisting of exclusively synthetic or a mixture of naturally-occurring and synthetic bases, any one of the foregoing linked to a polypeptide, and any one of the foregoing linked to any other molecular group or construct. Advantageously, the other molecular group or construct may be selected from the group consisting of nucleic acids,

polymeric substances, particularly beads, for example polystyrene beads, and magnetic or paramagnetic substances such as magnetic or paramagnetic beads.

The nucleic acid portion of the genetic element may comprise suitable regulatory sequences, such as those required for efficient expression of the gene product, for example promoters, enhancers, translational initiation sequences, polyadenylation sequences, splice sites and the like.

As will be apparent from the following, in many cases the polypeptide or other molecular group or construct is a ligand or a substrate which directly or indirectly binds to or reacts with the gene product in order to alter the optical properties of the genetic element. This allows the sorting of the genetic element on the basis of the activity of the gene product. The ligand or substrate can be connected to the nucleic acid by a variety of means that will be apparent to those skilled in the art (see, for example, Hermanson, 1996).

One way in which the nucleic acid molecule may be linked to a ligand or substrate is through biotinylation. This can be done by PCR amplification with a 5'-biotinylation primer such that the biotin and nucleic acid are covalently linked.

The ligand or substrate can be attached to the modified nucleic acid by a variety of means that will be apparent to those of skill in the art (see, for example, Hermanson, 1996). A biotinylated nucleic acid may be coupled to a polystyrene or paramagnetic microbead (0.02 to approx. 5.0 μm in diameter) that is coated with avidin or streptavidin, that will therefore bind the nucleic acid with very high affinity. This bead can be derivatised with substrate or ligand by any suitable method such as by adding biotinylated substrate or by covalent coupling.

Alternatively, a biotinylated nucleic acid may be coupled to avidin or streptavidin complexed to a large protein molecule such as thyroglobulin (669 Kd) or ferritin (440 Kd). This complex can be derivatised with substrate or ligand, for example by covalent coupling to the ε-amino group of lysines or through a non-covalent interaction such as biotin-avidin.

The substrate may be present in a form unlinked to the genetic element but containing an inactive "tag" that requires a further step to activate it such as photoactivation (e.g. of a "caged" biotin analogue, (Sundberg et al., 1995; Pirrung and Huang, 1996)). The catalyst to be selected then converts the substrate to product. The "tag" is then activated and the "tagged" substrate and/or product bound by a tag-binding molecule (e.g. avidin or streptavidin) complexed with the nucleic acid. The ratio of substrate to product attached to the nucleic acid *via* the "tag" will therefore reflect the ratio of the substrate and product in solution.

An alternative is to couple the nucleic acid to a product-specific antibody (or other product-specific molecule). In this scenario, the substrate (or one of the substrates) is present in each microcapsule unlinked to the genetic element, but has a molecular "tag" (for example biotin, DIG or DNP or a fluorescent group). When the catalyst to be selected converts the substrate to product, the product retains the "tag" and is then captured in the microcapsule by the product-specific antibody. In this way the genetic element only becomes associated with the "tag" when it encodes or produces an enzyme capable of converting substrate to product.

The terms "isolating", "sorting" and "selecting", as well as variations thereof, are used herein. Isolation, according to the present invention, refers to the process of separating an entity from a heterogeneous population, for example a mixture, such that it is free of at least one substance with which it was associated before the isolation process. In a preferred embodiment, isolation refers to purification of an entity essentially to homogeneity. Sorting of an entity refers to the process of preferentially isolating desired entities over undesired entities. In as far as this relates to isolation of the desired entities, the terms "isolating" and "sorting" are equivalent. The method of the present invention permits the sorting of desired genetic elements from pools (libraries or repertoires) of genetic elements which contain the desired genetic element. Selecting is used to refer to the process (including the sorting process) of isolating an entity according to a particular property thereof.

In a highly preferred application. the method of the present invention is useful for sorting libraries of genetic elements. The invention accordingly provides a method according to preceding aspects of the invention, wherein the genetic elements are isolated from a library of genetic elements encoding a repertoire of gene products. Herein, the terms "library", "repertoire" and "pool" are used according to their ordinary signification in the art, such that a library of genetic elements encodes a repertoire of gene products. In general, libraries are constructed from pools of genetic elements and have properties which facilitate sorting.

Initial selection of a genetic element from a genetic element library using the present invention will in most cases require the screening of a large number of variant genetic elements. Libraries of genetic elements can be created in a variety of different ways, including the following.

Pools of naturally occurring genetic elements can be cloned from genomic DNA or cDNA (Sambrook et al., 1989); for example, phage antibody libraries, made by PCR amplification repertoires of antibody genes from immunised or unimmunised donors have proved very effective sources of functional antibody fragments (Winter et al., 1994; Hoogenboom, 1997). Libraries of genes can also be made by encoding all (see for example Smith, 1985; Parmley and Smith, 1988) or part of genes (see for example Lowman et al., 1991) or pools of genes (see for example Nissim et al., 1994) by a randomised or doped synthetic oligonucleotide. Libraries can also be made by introducing mutations into a genetic element or pool of genetic elements 'randomly' by a variety of techniques *in vivo*, including; using mutator strains of bacteria such as *E. coli* mutD5 (Liao et al., 1986; Yamagishi et al., 1990; Low et al., 1996); using the antibody hypermutation system of B-lymphocytes (Yelamos et al., 1995). Random mutations can also be introduced both *in vivo* and *in vitro* by chemical mutagens, and ionising or UV irradiation (see Friedberg et al., 1995), or incorporation of mutagenic base analogues (Freese, 1959; Zaccolo et al., 1996). Random' mutations can also be introduced into genes *in vitro* during polymerisation for example by using error-prone polymerases (Leung et al., 1989).

Further diversification can be introduced by using homologous recombination either *in vivo* (see Kowalczykowski et al., 1994) or *in vitro* (Stemmer, 1994a; Stemmer, 1994b).

According to a further aspect of the present invention, therefore. there is provided a method of *in vitro* evolution comprising the steps of:

(a) selecting one or more genetic elements from a genetic element library according to the present invention;

(b) mutating the selected genetic element(s) in order to generate a further library of genetic elements encoding a repertoire to gene products; and

(c) iteratively repeating steps (a) and (b) in order to obtain a gene product with enhanced activity.

Mutations may be introduced into the genetic elements(s) as set forth above.

The genetic elements according to the invention advantageously encode enzymes, preferably of pharmacological or industrial interest, activators or inhibitors, especially of biological systems, such as cellular signal transduction mechanisms, antibodies and fragments thereof, and other binding agents (e.g. transcription factors) suitable for diagnostic and therapeutic applications. In a preferred aspect, therefore, the invention permits the identification and isolation of clinically or industrially useful products. In a further aspect of the invention, there is provided a product when isolated by the method of the invention.

The selection of suitable encapsulation conditions is desirable. Depending on the complexity and size of the library to be screened, it may be beneficial to set up the encapsulation procedure such that 1 or less than 1 genetic element is encapsulated per microcapsule. This will provide the greatest power of resolution. Where the library is larger and/or more complex, however, this may be impracticable; it may be preferable to encapsulate several genetic elements together and rely on repeated application of the method of the invention to achieve sorting of the desired activity. A combination of encapsulation procedures may be used to obtain the desired enrichment.

Theoretical studies indicate that the larger the number of genetic element variants created the more likely it is that a molecule will be created with the properties desired (see Perelson and Oster, 1979 for a description of how this applies to repertoires of antibodies). Recently it has also been confirmed practically that larger phage-antibody repertoires do indeed give rise to more antibodies with better binding affinities than smaller repertoires (Griffiths et al., 1994). To ensure that rare variants are generated and thus are capable of being selected, a large library size is desirable. Thus, the use of optimally small microcapsules is beneficial.

The largest repertoire created to date using methods that require an *in vivo* step (phage-display and LacI systems) has been a 1.6 x 10^{11} clone phage-peptide library which required the fermentation of 15 litres of bacteria (Fisch et al., 1996). SELEX experiments are often carried out on very large numbers of variants (up to 10^{15}).

Using the present invention, at a preferred microcapsule diameter of 2.6μm, a repertoire size of at least 10^{11} can be selected using 1ml aqueous phase in a 20 ml emulsion.

In addition to the genetic elements described above, the microcapsules according to the invention will comprise further components required for the sorting process to take place. Other components of the system will for example comprise those necessary for transcription and/or translation of the genetic element. These are selected for the requirements of a specific system from the following; a suitable buffer, an *in vitro* transcription/replication system and/or an *in vitro* translation system containing all the necessary ingredients, enzymes and cofactors, RNA polymerase, nucleotides, nucleic acids (natural or synthetic), transfer RNAs, ribosomes and amino acids, and the substrates of the reaction of interest in order to allow selection of the modified gene product.

A suitable buffer will be one in which all of the desired components of the biological system are active and will therefore depend upon the requirements of each specific reaction system. Buffers suitable for biological and/or chemical reactions are known in the art and recipes provided in various laboratory texts, such as Sambrook et al., 1989.

The *in vitro* translation system will usually comprise a cell extract, typically from bacteria (Zubay, 1973; Zubay, 1980; Lesley et al., 1991; Lesley, 1995), rabbit reticulocytes (Pelham and Jackson, 1976), or wheat germ (Anderson et al., 1983). Many suitable systems are commercially available (for example from Promega) including some which will allow coupled transcription/translation (all the bacterial systems and the reticulocyte and wheat germ TNT™ extract systems from Promega). The mixture of amino acids used may include synthetic amino acids if desired, to increase the possible number or variety of proteins produced in the library. This can be accomplished by charging tRNAs with artificial amino acids and using these tRNAs for the *in vitro* translation of the proteins to be selected (Ellman et al., 1991; Benner, 1994; Mendel et al., 1995).

After each round of selection the enrichment of the pool of genetic elements for those encoding the molecules of interest can be assayed by non-compartmentalised *in vitro* transcription/replication or coupled transcription-translation reactions. The selected pool is cloned into a suitable plasmid vector and RNA or recombinant protein is produced from the individual clones for further purification and assay.

In a preferred aspect, the internal environment of a microcapsule may be altered by addition of reagents to the oil phase of the emulsion. The reagents diffuse through the oil phase to the aqueous microcapsule environment. Preferably, the reagents are at least partly water-soluble, such that a proportion thereof is distributed from the oil phase to the aqueous microcapsule environment. Advantageously, the reagents are substantially insoluble in the oil phase. Reagents are preferably mixed into the oil phase by mechanical mixing, for example vortexing.

The reagents which may be added via the oil phase include substrates, buffering components, factors and the like. In particular, the internal pH of microcapsules may be altered *in situ* by adding acidic or basic components to the oil phase.

The invention moreover relates to a method for producing a gene product, once a genetic element encoding the gene product has been sorted by the method of the invention. Clearly, the genetic element itself may be directly expressed by conventional means to

produce the gene product. However, alternative techniques may be employed, as will be apparent to those skilled in the art. For example, the genetic information incorporated in the gene product may be incorporated into a suitable expression vector, and expressed therefrom.

The invention also describes the use of conventional screening techniques to identify compounds which are capable of interacting with the gene products identified by the first aspect of the invention. In preferred embodiments, gene product encoding nucleic acid is incorporated into a vector, and introduced into suitable host cells to produce transformed cell lines that express the gene product. The resulting cell lines can then be produced for reproducible qualitative and/or quantitative analysis of the effect(s) of potential drugs affecting gene product function. Thus gene product expressing cells may be employed for the identification of compounds, particularly small molecular weight compounds, which modulate the function of gene product. Thus host cells expressing gene product are useful for drug screening and it is a further object of the present invention to provide a method for identifying compounds which modulate the activity of the gene product, said method comprising exposing cells containing heterologous DNA encoding gene product, wherein said cells produce functional gene product, to at least one compound or mixture of compounds or signal whose ability to modulate the activity of said gene product is sought to be determined, and thereafter monitoring said cells for changes caused by said modulation. Such an assay enables the identification of modulators, such as agonists, antagonists and allosteric modulators, of the gene product. As used herein, a compound or signal that modulates the activity of gene product refers to a compound that alters the activity of gene product in such a way that the activity of the gene product is different in the presence of the compound or signal (as compared to the absence of said compound or signal).

Cell-based screening assays can be designed by constructing cell lines in which the expression of a reporter protein, i.e. an easily assayable protein, such as β-galactosidase, chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP) or luciferase, is dependent on gene product. Such an assay enables the detection of compounds that directly modulate gene product function, such as compounds that antagonise gene

product, or compounds that inhibit or potentiate other cellular functions required for the activity of gene product.

The present invention also provides a method to exogenously affect gene product dependent processes occurring in cells. Recombinant gene product producing host cells, e.g. mammalian cells, can be contacted with a test compound, and the modulating effect(s) thereof can then be evaluated by comparing the gene product-mediated response in the presence and absence of test compound, or relating the gene product-mediated response of test cells, or control cells (i.e., cells that do not express gene product), to the presence of the compound.

In a further aspect, the invention relates to a method for optimising a production process which involves at least one step which is facilitated by a polypeptide. For example, the step may be a catalytic step, which is facilitated by an enzyme. Thus, the invention provides a method for preparing a compound or compounds comprising the steps of:

(a) providing a synthesis protocol wherein at least one step is facilitated by a polypeptide;

(b) preparing genetic elements encoding variants of the polypeptide which facilitates this step, the expression of which may result, directly or indirectly, in the modification of the optical properties of the genetic elements;

(c) compartmentalising genetic elements into microcapsules;

(d) expressing the genetic elements to produce their respective gene products within the microcapsules;

(e) sorting the genetic elements which produce polypeptide gene product(s) having the desired activity using the changed optical properties of the genetic elements; and

(f) preparing the compound or compounds using the polypeptide gene product identified in (g) to facilitate the relevant step of the synthesis.

By means of the invention, enzymes involved in the preparation of a compound may be optimised by selection for optimal activity. The procedure involves the preparation of variants of the polypeptide to be screened, which equate to a library of polypeptides as

refereed to herein. The variants may be prepared in the same manner as the libraries discussed elsewhere herein.

(B) SELECTION PROCEDURES

The system can be configured to select for RNA, DNA or protein gene product molecules with catalytic, regulatory or binding activity.

(i) SELECTION FOR BINDING

In the case of selection for a gene product with affinity for a specific ligand the genetic element may be linked to the gene product in the microcapsule *via* the ligand. Only gene products with affinity for the ligand will therefore bind to the genetic element and only those genetic elements with gene product bound *via* the ligand will acquire the changed optical properties which enable them to be retained in the selection step. In this embodiment, the genetic element will thus comprise a nucleic acid encoding the gene product linked to a ligand for the gene product.

The change in optical properties of the genetic element after binding of the gene product to the ligand may be induced in a variety of ways, including:

(1) the gene product itself may have distinctive optical properties, for example, it is fluorescent (e.g. green fluorescent protein, (Lorenz *et al.*, 1991)).

(2) the optical properties of the gene product may be modified on binding to the ligand, for example, the fluorescence of the gene product is quenched or enhanced on binding (Guixe et al., 1998; Qi and Grabowski, 1998)

(3) the optical properties of the ligand may be modified on binding of the gene product, for example, the fluorescence of the ligand is quenched or enhanced on binding (Voss, 1993; Masui and Kuramitsu, 1998).

(4) the optical properties of both ligand and gene product are modified on binding, for example, there can be a fluorescence resonance energy transfer (FRET) from ligand to gene product (or *vice versa*) resulting in emmission at the "acceptor" emmission

wavelength when excitation is at the "donor" absoption wavelength (Heim & Tsien. 1996; Mahajan *et al.*, 1998; Miyawaki *et al.*, 1997).

In this embodiment, it is not necessary for binding of the gene product to the genetic element *via* the ligand to directly induce a change in optical properties. All the gene products to be selected can contain a putative binding domain, which is to be selected for, and a common feature - a tag. The genetic element in each microcapsule is physically linked to the ligand. If the gene product produced from the genetic element has affinity for the ligand, it will bind to it and become physically linked to the same genetic element that encoded it, resulting in the genetic element being 'tagged'. At the end of the reaction, all of the microcapsules are combined, and all genetic elements and gene products pooled together in one environment. Genetic elements encoding gene products exhibiting the desired binding can be selected by adding reagents which specifically bind to, or react specifically with, the "tag" and thereby induce a change in the optical properties of the genetic element allowing there sorting. For example, a fluorescently-labelled anti-"tag" antibody can be used, or an anti-"tag" antibody followed by a second fluorescently labelled antibody which binds the first.

In an alternative embodiment, genetic elements may be sorted on the basis that the gene product, which binds to the ligand, merely hides the ligand from, for example, further binding partners which would otherwise modify the optical properties of the genetic element. In this case genetic elements with unmodified optical properties would be selected.

In an alternative embodiment, the invention provides a method according to the first aspect of the invention, wherein in step (b) the gene products bind to genetic elements encoding them. The gene products together with the attached genetic elements are then sorted as a result of binding of a ligand to gene products having the desired binding activity. For example, all gene products can contain an invariant region which binds covalently or non-covalently to the genetic element, and a second region which is diversified so as to generate the desired binding activity.

In an alternative embodiment, the ligand for the gene product is itself encoded by the genetic element and binds to the genetic element. Stated otherwise, the genetic element encodes two (or indeed more) gene products, at least one of which binds to the genetic element, and which can potentially bind each other. Only when the gene products interact in a microcapsule is the genetic element modified in a way that ultimately results in a change in a change in its optical properties that enables it to be sorted. This embodiment, for example, isused to search gene libraries for pairs of genes encoding pairs of proteins which bind each other.

Fluorescence may be enhanced by the use of Tyramide Signal Amplification (TSA™) amplification to make the genetic elements fluorescent. This involves peroxidase (linked to another protein) binding to the genetic elements and catalysing the conversion of fluorescein-tyramine in to a free radical form which then reacts (locally) with the genetic elements. Methods for performing TSA are known in the art, and kits are available commercially from NEN.

TSA may be configured such that it results in a direct increase in the fluorescence of the genetic element, or such that a ligand is attached to the genetic element which is bound by a second fluorescent molecule, or a sequence of molecules, one or more of which is fluorescent.

(ii) SELECTION FOR CATALYSIS

When selection is for catalysis, the genetic element in each microcapsule may comprise the substrate of the reaction. If the genetic element encodes a gene product capable of acting as a catalyst, the gene product will catalyse the conversion of the substrate into the product. Therefore, at the end of the reaction the genetic element is physically linked to the product of the catalysed reaction.

It may also be desirable, in some cases, for the substrate not to be a component of the genetic element. In this case the substrate would contain an inactive "tag" that requires a further step to activate it such as photoactivation (e.g. of a "caged" biotin analogue, (Sundberg et al., 1995; Pirrung and Huang, 1996)). The catalyst to be selected then converts the substrate to product. The "tag" is then activated and the "tagged" substrate

and/or product bound by a tag-binding molecule (e.g. avidin or streptavidin) complexed with the nucleic acid. The ratio of substrate to product attached to the nucleic acid *via* the "tag" will therefore reflect the ratio of the substrate and product in solution.

The optical properties of genetic elements with product attached and which encode gene products with the desired catalytic activity can be modified by either:

(1) the product-genetic element complex having characteristic optical properties not found in the substrate-genetic element complex, due to, for example;

(a) the substrate and product having different optical properties (many fluorogenic enzyme substrates are available commercially (see for example Haugland, 1996) including substrates for glycosidases, phosphatases, peptidases and proteases (Craig et al., 1995; Huang et al., 1992; Brynes et al., 1982; Jones et al., 1997; Matayoshi et al., 1990; Wang et al., 1990)), or

(b) the substrate and product having similar optical properties, but only the product, and not the substrate binds to, or reacts with, the genetic element;

(2) adding reagents which specifically bind to, or react with, the product and which thereby induce a change in the optical properties of the genetic elements allowing their sorting (these reagents can be added before or after breaking the microcapsules and pooling the genetic elements). The reagents ;

(a) bind specifically to, or react specifically with, the product, and not the substrate, if both substrate and product are attached to the genetic element, or

(b) optionally bind both substrate and product if only the product, and not the substrate binds to, or reacts with, the genetic element.

The pooled genetic elements encoding catalytic molecules can then be enriched by selecting for the genetic elements with modified optical properties.

An alternative is to couple the nucleic acid to a product-specific antibody (or other product-specific molecule). In this scenario, the substrate (or one of the substrates) is present in each microcapsule unlinked to the genetic element, but has a molecular "tag" (for example biotin, DIG or DNP or a fluorescent group). When the catalyst to be selected converts the substrate to product, the product retains the "tag" and is then

captured in the microcapsule by the product-specific antibody. In this way the genetic element only becomes associated with the "tag" when it encodes or produces an enzyme capable of converting substrate to product. When all reactions are stopped and the microcapsules are combined, the genetic elements encoding active enzymes will be "tagged" and may already have changed optical properties, for example, if the "tag" was a fluorescent group. Alternatively, a change in optical properties of "tagged" genes can be induced by adding a fluorescently labelled ligand which binds the "tag" (for example fluorescently-labelled avidin/streptavidin, an anti-"tag" antibody which is fluorescent, or a non-fluorescent anti-"tag" antibody which can be detected by a second fluorescently-labelled antibody).

Alternatively, selection may be performed indirectly by coupling a first reaction to subsequent reactions that takes place in the same microcapsule. There are two general ways in which this may be performed. In a first embodiment, the product of the first reaction is reacted with, or bound by, a molecule which does not react with the substrate of the first reaction. A second, coupled reaction will only proceed in the presence of the product of the first reaction. A genetic element encoding a gene product with a desired activity can then be purified by using the properties of the product of the second reaction to induce a change in the optical properties of the genetic element as above.

Alternatively, the product of the reaction being selected may be the substrate or cofactor for a second enzyme-catalysed reaction. The enzyme to catalyse the second reaction can either be translated in situ in the microcapsules or incorporated in the reaction mixture prior to microencapsulation. Only when the first reaction proceeds will the coupled enzyme generate a product which can be used to induce a change in the optical properties of the genetic element as above.

This concept of coupling can be elaborated to incorporate multiple enzymes, each using as a substrate the product of the previous reaction. This allows for selection of enzymes that will not react with an immobilised substrate. It can also be designed to give increased sensitivity by signal amplification if a product of one reaction is a catalyst or a cofactor for a second reaction or series of reactions leading to a selectable product (for example,

see Johannsson and Bates, 1988; Johannsson, 1991). Furthermore an enzyme cascade system can be based on the production of an activator for an enzyme or the destruction of an enzyme inhibitor (see Mize et al., 1989). Coupling also has the advantage that a common selection system can be used for a whole group of enzymes which generate the same product and allows for the selection of complicated chemical transformations that cannot be performed in a single step.

Such a method of coupling thus enables the evolution of novel "metabolic pathways" *in vitro* in a stepwise fashion, selecting and improving first one step and then the next. The selection strategy is based on the final product of the pathway, so that all earlier steps can be evolved independently or sequentially without setting up a new selection system for each step of the reaction.

Expressed in an alternative manner, there is provided a method of isolating one or more genetic elements encoding a gene product having a desired catalytic activity, comprising the steps of:

(1) expressing genetic elements to give their respective gene products;

(2) allowing the gene products to catalyse conversion of a substrate to a product, which may or may not be directly selectable, in accordance with the desired activity;

(3) optionally coupling the first reaction to one or more subsequent reactions, each reaction being modulated by the product of the previous reactions, and leading to the creation of a final, selectable product;

(4) linking the selectable product of catalysis to the genetic elements by either:

a) coupling a substrate to the genetic elements in such a way that the product remains associated with the genetic elements, or

b) reacting or binding the selectable product to the genetic elements by way of a suitable molecular "tag" attached to the substrate which remains on the product, or

c) coupling the selectable product (but not the substrate) to the genetic elements by means of a product-specific reaction or interaction with the product; and

(5) selecting the product of catalysis, together with the genetic element to which it is bound, either by means of its characteristic optical properties, or by adding reagents

which specifically bind to, or react specifically with, the product and which thereby induce a change in the optical properties of the genetic elements wherein steps (1) to (4) each genetic element and respective gene product is contained within a microcapsule.

(iii) SELECTING FOR ENZYME SUBSTRATE SPECIFICITY/SELECTIVITY

Genetic elements encoding enzymes with substrate specificity or selectivity can be specifically enriched by carrying out a positive selection for reaction with one substrate and a negative selection for reaction with another substrate. Such combined positive and negative selection pressure should be of great importance in isolating regio-selective and stereo-selective enzymes (for example, enzymes that can distinguish between two enantiomers of the same substrate). For example, two substrates (e.g. two different enantiomers) are each labelled with different tags (e.g. two different fluorophores) such that the tags become attached to the genetic element by the enzyme-catalysed reaction. If the two tags confer different optical properties on the genetic element the substrate specificity of the enzyme can be determined from the optical properties of the genetic element and those genetic elements encoding gene products with the wrong (or no) specificity rejected. Tags conferring no change in optical activity can also be used if tag-specific ligands with different optical properties are added (e.g. tag-specific antibodies labelled with different fluorophores).

(iv) SELECTION FOR REGULATION

A similar system can be used to select for regulatory properties of enzymes.

In the case of selection for a regulator molecule which acts as an activator or inhibitor of a biochemical process, the components of the biochemical process can either be translated *in situ* in each microcapsule or can be incorporated in the reaction mixture prior to microencapsulation. If the genetic element being selected is to encode an activator, selection can be performed for the product of the regulated reaction, as described above in

connection with catalysis. If an inhibitor is desired, selection can be for a chemical property specific to the substrate of the regulated reaction.

There is therefore provided a method of sorting one or more genetic elements coding for a gene product exhibiting a desired regulatory activity, comprising the steps of:

(1) expressing genetic elements to give their respective gene products;

(2) allowing the gene products to activate or inhibit a biochemical reaction, or sequence of coupled reactions, in accordance with the desired activity, in such a way as to allow the generation or survival of a selectable molecule;

(3) linking the selectable molecule to the genetic elements either by

a) having the selectable molecule, or the substrate from which it derives, attached to the genetic elements, or

b) reacting or binding the selectable product to the genetic elements, by way of a suitable molecular "tag" attached to the substrate which remains on the product, or

c) coupling the product of catalysis (but not the substrate) to the genetic elements, by means of a product-specific reaction or interaction with the product;

(4) selecting the selectable product, together with the genetic element to which it is bound, either by means of its characteristic optical properties, or by adding reagents which specifically bind to, or react specifically with, the product and which thereby induce a change in the optical properties of the genetic elements wherein steps (1) to (3) each genetic element and respective gene product is contained within a microcapsule.

(v) SELECTION FOR OPTICAL PROPERTIES OF THE GENE PRODUCT

It is possible to select for inherent optical properties of gene products if, in the microcapsules, the gene product binds back to the genetic element, for example through a common element of the gene product which binds to a ligand which is part of the genetic element. After pooling the genetic elements they can then be sorted using the optical properties of the bound gene products. This embodiment can be used, for example, to select variants of green fluorescent protein (GFP) (Cormack *et al.*, 1996; Delagrave *et al.*,

1995; Ehrig *et al.*, 1995), with improved fluorescence and/or novel absoption and emmission spectra.

(vi) FLOW SORTING OF GENETIC ELEMENTS

In a preferred embodiment of the invention the genetic elements will be sorted by flow cytometry. A variety of optical properties can be used to trigger sorting, including light scattering (Kerker, 1983) and fluorescence polarisation (Rolland et al., 1985). In a highly preferred embodiment the difference in optical properties of the genetic elements will be a difference in fluorescence and the genetic elements will be sorted using a fluorescence activated cell sorter (Norman, 1980; Mackenzie and Pinder, 1986), or similar device. In an especially preferred embodiment the genetic element comprises of a nonfluorescent nonmagnetic (e.g. polystyrene) or paramagnetic microbead (see Fornusek and Vetvicka, 1986), optimally 0.6 to 1.0 μm diameter, to which are attached both the gene and the groups involved in generating a fluorescent signal:

(1) commercially available fluorescence activated cell sorting equipment from established manufacturers (e.g. Becton-Dickinson, Coulter) allows the sorting of up to 10^8 genetic elements (events) per hour;

(2) the fluorescence signal from each bead corresponds tightly to the number of fluorescent molecules attached to the bead. At present as little as few hundred fluorescent molecules per particle can be quantitatively detected;

(3) the wide dynamic range of the fluorescence detectors (typically 4 log units) allows easy setting of the stringency of the sorting procedure, thus allowing the recovery of the optimal number of genetic elements from the starting pool (the gates can be set to separate beads with small differences in fluorescence or to only separate out beads with large differences in fluorescence, dependant on the selection being performed;

(4) commercially available fluorescence-activated cell sorting equipment can perform simultaneous excitation at up to two different wavelengths and detect fluorescence at up to four different wavelengths (Shapiro, 1983) allowing positive and negative selections to be performed simultaneously by monitoring the labelling of the genetic element with two (or more) different fluorescent markers, for example, if two alternative substrates for an enzyme (e.g. two different enantiomers) are labelled with

different fluorescent tags the genetic element can labelled with different fluorophores dependent on the substrate used and only genes encoding enzymes with enantioselectivity selected.

(5) highly uniform derivatised and non-derivatised nonmagnetic and paramagnetic microparticles (beads) are commercially available from many sources (e.g. Sigma, and Molecular Probes) (Fornusek and Vetvicka, 1986).

(vii) MULTI-STEP PROCEDURE

It will be also be appreciated that according to the present invention, it is not necessary for all the processes of transcription/replication and/or translation, and selection to proceed in one single step, with all reactions taking place in one microcapsule. The selection procedure may comprise two or more steps. First, transcription/replication and/or translation of each genetic element of a genetic element library may take place in a first microcapsule. Each gene product is then linked to the genetic element which encoded it (which resides in the same microcapsule), for example *via* a gene product-specific ligand such as an antibody. The microcapsules are then broken, and the genetic elements attached to their respective gene products optionally purified. Alternatively, genetic elements can be attached to their respective gene products using methods which do not rely on encapsulation. For example phage display (Smith, G.P.,1985), polysome display (Mattheakkis et al., 1994), RNA-peptide fusion (Roberts and Szostak, 1997) or lac repressor peptide fusion (Cull, et al., 1992).

In the second step of the procedure, each purified genetic element attached to its gene product is put into a second microcapsule containing components of the reaction to be selected. This reaction is then initiated. After completion of the reactions, the microcapsules are again broken and the modified genetic elements are selected. In the case of complicated multistep reactions in which many individual components and reaction steps are involved, one or more intervening steps may be performed between the initial step of creation and linking of gene product to genetic element, and the final step of generating the selectable change in the genetic element.

If necessary, release of the gene product from the genetic element within a secondary microcapsule can be achieved in a variety of ways, including by specific competition by a low-molecular weight product for the binding site or cleavage of a linker region joining the binding domain of the gene product from the catalytic domain either enzymatically (using specific proteases) or autocatalytically (using an integrin domain).

(viii) SELECTION BY ACTIVATION OF REPORTER GENE EXPRESSION IN SITU

The system can be configured such that the desired binding, catalytic or regulatory activity encoded by a genetic element leads, directly or indirectly to the activation of expression of a "reporter gene" that is present in all microcapsules. Only gene products with the desired activity activate expression of the reporter gene. The activity resulting from reporter gene expression allows the selection of the genetic element (or of the compartment containing it) by any of the methods described herein.

For example, activation of the reporter gene may be the result of a binding activity of the gene product in a manner analogous to the "two hybrid system" (Fields and Song, 1989). Activation can also result from the product of a reaction catalysed by a desirable gene product. For example, the reaction product can be a transcriptional inducer of the reporter gene. For example arabinose may be used to induce transcription from the araBAD promoter. The activity of the desirable gene product can also result in the modification of a transcription factor, resulting in expression of the reporter gene. For example, if the desired gene product is a kinase or phosphatase the phosphorylation or dephosphorylation of a transcription factor may lead to activation of reporter gene expression.

(ix) AMPLIFICATION

According to a further aspect of the present invention the method comprises the further step of amplifying the genetic elements. Selective amplification may be used as a means to enrich for genetic elements encoding the desired gene product.

In all the above configurations, genetic material comprised in the genetic elements may be amplified and the process repeated in iterative steps. Amplification may be by the polymerase chain reaction (Saiki et al., 1988) or by using one of a variety of other gene amplification techniques including; Qb replicase amplification (Cahill, Foster and Mahan, 1991; Chetverin and Spirin, 1995; Katanaev, Kurnasov and Spirin, 1995); the ligase chain reaction (LCR) (Landegren et al., 1988; Barany, 1991); the self-sustained sequence replication system (Fahy, Kwoh and Gingeras, 1991) and strand displacement amplification (Walker et al., 1992).

Various aspects and embodiments of the present invention are illustrated in the following examples. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

All documents mentioned in the text are incorporated by reference.

EXAMPLES

Example 1.

Enzymes can be expressed from genes in solution and genes attached to paramagnetic microbeads with identical efficiency.

One format for the selection of genetic elements by using a change in their optical properties is one in which the genetic element comprises a microbead to which the gene is attached. Here it is shown how a gene for an enzyme (*E. coli* dihydrofolate reductase) can be linked to a paramagnetic bead and is translated *in vitro* just as efficiently as in solution.

The *E. coli folA* gene encoding dihydrofolate reductase (DHFR) is PCR-amplified using oligonucleotides EDHFRFo and EDHFRBa. This DNA is then cloned into the pGEM-4Z vector (Promega) digested with HindIII and KpnI downstream of the lac promoter and the T7 RNA polymerase promoter. The oligonucleotide EDHFRBa appends the efficient phage T7 gene 10 translational start site upstream of the DHFR start codon.

DNA sequencing identifies a clone which has the correct nucleotide sequence. Bacteria transformed with this clone (pGEM-folA) are found to over-express active DHFR (driven from the lac promoter) when induced with IPTG.

The *fol*A gene in pGEM-folA plasmid is then PCR-amplified using primers folA-FW and folA-BW, the resulting DNA fragment in HindIII and XhoI digested and subcloned into HindIII/XhoI- digested pET23a expression vector (Novagen) to give construct pET23a/folA. The sequence of PCR-amplified folA gene was verified by sequencing.

pET23a/folA was further amplified with 5'-biotinylated primers pETfor.b and pETrev.b and radio-labelled by including 10 µCi α^{35}S-dATP (Amersham Pharmacia Biotech, U.K.) in the PCR mix. The resulting 1765 bp double biotinylated fragment T7-folA was gel purified using a Qiagen kit and quantified spectrophotometrically. The specific activity of the product was 210000 CPM/pmol T7-folA DNA, as measured on the Beckman LS6000SC scintillation counter. 10 nM and 1 nM dilutions of this DNA were made in 1 mg/ml HindIII digested lambda DNA to eliminate non-specific binding to the plastic.). This PCR fragment was used thereafter to program a prokaryotic *in vitro* coupled transcription/translation system designed for linear templates (Lesley, Brow and Burgess, 1991). A commercial preparation of this system is used (*E. coli* S30 Extract System for Linear Templates; Promega) supplemented with T7 RNA polymerase (10^3 units).

The DNA fragment is bound to streptavidin-paramagnetic beads (0.74 µm diameter Sera-Mag beads, biotin-binding capacity 46nmol/mg, Seradyn, USA), partially precoated with biotinylated protein A (Sigma). 2 µl of 80 µM biotinylated protein A is added to 100 µl (1 mg) beads, allowed to bind at room temperature for 1 hour, washed once and coated for one hour at room temperature with rabbit IgG (10 µl 1 mg/ml antibody per 1 mg beads in TBS/0.1% Tween-20 (TBST)). Beads were thereafter washed twice with TBS/T before radiolabeled biotinylated T7-folA DNA was added and allowed to bind for 1 hour at room temperature. The amount of bound T7-folA DNA was calculated by counting the radioactivity bound to an aliquot of beads. ~50% of the total DNA was bound.

DNA fragments bound on beads or unbound DNA fragment are added directly to the S30 Extract System. Reactions are incubated for 2 hours at 37 °C.

Dihydrofolate reductase activity is assayed by spectrophotometrically monitoring the oxidation of NADPH to NADP at 340nm over a 10 minute time course as described by (Williams et al., 1979; Ma et al., 1993). 2 µl of each quenched *in vitro* translation reaction is added to 150µl Buffer A (100 mM Imidazole, pH 7.0, 10 mM β-mercaptoethanol) and 20µl 1mM NADPH. 20µl dihydrofolate (1mM)(H_2F) is added after 1 minute and the reaction monitored at 340nm using a ThermoMax microplate reader (Molecular Devices). Activity is calculated by initial velocities under $So>>K_M$ conditions (υmax).

There is no significant difference in the amount of active DHFR produced if the DNA is free, or attached *via* terminal biotins to a streptavidin coated bead (see Figure 1).

Example 2.

A fluorescent protein (GFP) can be translated *in vitro* from genes attached to single microbeads encapsulated in the aqueous compartments of a water-in-oil emulsion and the translated gene-product bound back to the microbeads making them fluorescent.

One format for the selection of genetic elements is where the genetic element comprises a gene linked to a microbead and the product is coupled back onto the microbead within the microcapsule resulting directly, or indirectly, in a change in the optical properties of the microbead which allows it to be sorted.

Here it is shown that a fluorescent protein (green fluorescent protein or GFP) can be transcribed and translated *in vitro* from genes attached to single microbeads encapsulated in the aqueous compartments of a water-in-oil emulsion and the translated gene-product bound back the microbeads making them fluorescent.

The GFP in pBS/GFP6 plasmid (Siemering et al.. 1996) was PCR-amplified using primers GFP-FW and GFP-BW, the resulting DNA fragment in HindIII and XhoI digested and subcloned into HindIII/XhoI- digested pET23a expression vector (Novagen) to give construct pET23a/GFP. The sequence of PCR-amplified GFP gene was verified by sequencing. pET23a/GFP was further amplified with 5'-biotinylated primers pETfor.b and pETrev.b. The resulting 2038 bp double biotinylated fragment T7-GFP was gel purified using a Qiagen kit and quantified spectrophotometrically. 10 nM and 1 nM dilutions of this DNA were made in 1 mg/ml HindIII digested lambda DNA to eliminate non-specific binding to the plastic.). This PCR fragment was used thereafter to program a prokaryotic *in vitro* coupled transcription/translation system designed for linear templates (Lesley, Brow and Burgess, 1991). A commercial preparation of this system is used (*E. coli* S30 Extract System for Linear Templates; Promega) supplemented with T7 RNA polymerase (10^3 units).

As a control, a biotinylated 1765bp DNA fragment T7-folA (synthesised by PCR as in example 1) was used to program the synthesis of the non-fluorescent protein DHFR.

150 µl ProActive streptavidin-coated paramagnetic beads (Bangs Laboratories, 2×10^7beads/µl) were suspended in 5mM Tris 7.4/1M NaCl/0.1% Tween20 and split into three aliquots of 50 µl. 0.5 µl of 0.2 µM DNA (T7-folA or T7-GFP) was added to each aliquot of beads, incubated at 43°C for 15 min, washed three times in 25 mM NaH_2PO_4, 125 mM NaCl, 0.1% Tween20, pH 7.0 (PBS/0.1% Tween20), resuspended in 40 µl TBST and 10 µl 80 µM biotinylated protein A (Sigma) was added (to give final concentration of 15 µM). After incubation for 30 minutes at room temperature, the beads were washed three times in PBS/0.1% Tween20 and resuspended in 20 µl 1:10 dilution rabbit anti-GFP polyclonal antibody (Clontech) or 1 mg/ml unimmunised rabbit IgG (Sigma). After incubation for 30 minutes at room temperature, the beads were washed three times in PBS/0.1% Tween20 and resuspended in 15 µl of S30 premix from an *E. coli* S30 Extract System for Linear Templates (Promega), sonicated for one minute in a sonication bath, then the rest of the S30 *in vitro* translation mixture was added (on ice) and supplemented with T7 RNA polymerase (10^3 units).

The 50 μl ice-cooled *in vitro* translation reactions were added gradually (in 5 aliquots of 10 μl over ~2 minutes) to 0.95 ml of ice-cooled oil-phase (freshly prepared by dissolving 4.5% (v/v) Span 80 (Fluka) in mineral oil (Sigma, #M-5904) followed by 0.5% (v/v) Tween 80 (SigmaUltra; #P-8074)in a 5 ml Costar Biofreeze Vial (#2051)) whilst stirring with a magnetic bar (8x3 mm with a pivot ring; Scientific Industries International, Loughborough, UK). Stirring (at 1150 rpm) was continued for an additional 3 minutes on ice. Reactions were then incubated 3h at 32°C.

2 μl of emulsion were spread on a microscope slide beneath a 13 mm round cover slip and visualised using a 20xNeofluar objective on an Axioplan microscope (Zeiss) equipped with an RTEA CCD-1300-Y CCD camera (Princeton Instruments). Standard excitation and emission filters for fluorescein were used and images were processed with IPLab software.

As can be seen from Figure 2 the GFP translated from genes attached to single microbeads encapsulated in the aqueous compartments of the emulsions is bound to the microbeads *in situ* when the microbeads are coated with an anti-GFP antibody. This binding is observed as concentration of fluorescence on the beads by epifluorescence microscopy. No bead fluorescence is observed when either the GFP gene or the anti-GFP antibody are missing.

Example 3.

A fluorescent protein (GFP) can be translated *in vitro* from genes attached to single microbeads encapsulated in the aqueous compartments of a water-in-oil emulsion, the translated gene-product bound back the microbeads and the increased fluorescence of the microbeads detected by flow cytometry.

150 μl streptavidin-coated polystyrene beads (diameter 1 μM; Bangs Laboratories, $2x10^7$ beads/μl) were suspended in 5mM Tris 7.4/1M NaCl/0.1% Tween20 and split into three aliquots of 50 μl. 0.5 μl of 0.2 μM DNA (T7-folA or T7-GFP) was added to each aliquot of beads, incubated at 43°C for 15 min, washed three times in 25 mM NaH_2PO_4, 125 mM NaCl, 0.1% Tween20, pH 7.0 (PBS/0.1% Tween20), resuspended in 40 μl TBST and 10

μl 80 μM biotinylated protein A (Sigma)was added (to give final concentration of 15 μM). After incubation for 30 minutes at room temperature, the beads were washed three times in PBS/0.1% Tween20 and resuspended in 20 μl 1:10 dilution rabbit anti-GFP polyclonal antibody (Clontech) or 1 mg/ml unimmunised rabbit IgG (Sigma). After incubation for 30 minutes at room temperature, the beads were washed three times in PBS/0.1% Tween20 and resuspended in 15 μl of S30 premix from an *E. coli* S30 Extract System for Linear Templates (Promega), sonicated for one minute in a sonication bath, then the rest of the S30 *in vitro* translation mixture was added (on ice) and supplemented with T7 RNA polymerase (10^3 units).The 50 μl ice-cooled in vitro translation reactions were added gradually (in 5 aliquots of 10 μl over ~2 minutes) to 0.95 ml of ice-cooled oil-phase (freshly prepared by dissolving 4.5% (v/v) Span 80 (Fluka) in mineral oil (Sigma, #M-5904) followed by 0.5% (v/v) Tween 80 (SigmaUltra; #P-8074)in a 5 ml Costar Biofreeze Vial (#2051)) whilst stirring with a magnetic bar (8x3 mm with a pivot ring; Scientific Industries International, Loughborough, UK). Stirring (at 1150 rpm) was continued for an additional 3 minutes on ice. Reactions were then incubated 3h at 32°C.To recover the reaction mixtures, the emulsions were spun at 3,000 g for 5 minutes and the oil phase removed leaving the concentrated (but still intact) emulsion at the bottom of the vial. PBS and 2 ml of water-saturated ether were added and the mixture was vortexed, centrifuged briefly, and the ether phase removed. Beads were washed twice with PBS and finally resuspended at 10^8 beads/ml in PBS. 10^4 beads were analysed using a FACScalibur flow cytometer (Becton Dickinson) using excitation at 488 nm and the fluorescein emission filter. The GFP translated from genes attached to single microbeads encapsulated in the aqueous compartments of the emulsions is bound to the microbeads *in situ* when the microbeads are coated with an anti-GFP antibody. The binding of GFP to the microbeads makes them fluorescent (Fig. 2), and those beads with GFP bound can be clearly distinguished from those which do not by flow cytometry (Figure 3).

Example 4

The product of an enzyme catalysed reaction can be captured on paramagnetic beads and beads derivatised with product identified by flow cytometry.

A reaction catalysed by the enzyme human glutathione *S*-transferase M2-2 (GST M2-2) was performed to generate a biotinylated product (Figure 4). The two substrates used were 1-chloro-2,4-dinitrobenzene (CDNB; Sigma) and reduced biotinylated-glutathione (Biotin-GSH). The product generated (Biotin-GS-DNP) has biotin at one end to enable coupling to streptavidin-coated paramagnetic microparticles and a 2,4-dinitrophenol (DNP) group which can be bound by an anti-DNP antibody.

Biotin-GSH was synthesised by adding 100 mg biotinamidocaproate N-hydroxysuccinimide ester (biotin-NHS; Sigma) in 1 ml DMF to a solution of oxidised glutathione (Fluka) in 1 ml water, 30 μl 12.5N NaOH plus 1 ml DMF. The biotin-NHS was added, on ice, in 100 μl aliquots over 20 minutes. The pH was then adjusted to 7.0 with 1N NaOH. The syrup-like precipitate which formed during the reaction was dissolved by warming to room temperature, vortexing and adding 300 μl water. Stirring was continued for 2 hours at room temperature, the pH brought back to 7.0 by adding 1N NaOH and stirred overnight at room temperature. NaOH was then used to bring the pH back to 7.5, the reaction stirred a further 30 minutes at room temperature and then incubated 30 minutes more after adding 500 μl 1M DTT. The solvents were evaporated under vacuum and the product purified by reverse-phase HPLC using a C8 column and a gradient of 10-40% Acetonitrile, 0.1% TFA. Biotin-GS-DNP was synthesised enzymatically in a 100 μl reaction containing 1 μg purified recombinant GST M2-2, 500 μM CDNB and 200 μM Biotin-GSH in 0.1 M KH_2PO_4, 1 mM EDTA, pH6.5. Incubation was for 1 hour at 25°C. The reaction went essentially to completion as judged by following the increase in absorbance at 340 nm. Control reactions were also performed 1) with no GST, 2) with no CDNB, and 3) with no biotin-GSH. Reactions were diluted 200 times (giving a final concentration of 1 μM biotin) into 5 mM Tris-HCl, 0.5 mM EDTA, 1.0 M NaCl, pH7.4 (B/W buffer). 50 μl of the diluted reactions were mixed with 50 μl B/W buffer containing 29.3 μg (10^8 microparticles) 0.737 μm diameter Sera-Mag™ streptavidin-coated magnetic microparticles (MG-SA; Seradyn) and incubated 1 hour at room temperature. Microparticles were separated in a microtitre plate (Falcon 3911) using a magnet (Dynal MPC-96) and washed three times with 10 mM Tris-HCl, 1 mM EDTA, 2.0 M NaCl, pH7.4 (2xB/W buffer), then twice with PBS, 0.1% Tween 20. The microparticles were resuspended in a 1:2500 dilution of the mouse anti-dinitrophenol monoclonal antibody SPE 21-11 (a gift from Prof. Zelig Eshhar) in PBS/0.1% Tween 20

and incubated 45 minutes at room temperature. The microparticles were washed three times in PBS/0.1% Tween 20, resuspended in PBS/0.1% Tween 20 containing 15 μg/ml fluorescein (FITC)-conjugated F(ab')2 fragment goat anti-mouse IgG, F(ab')2 fragment (Jackson; 115-096-006) and incubated 30 minutes at room temperature. The microparticles were washed four times in PBS/0.1% Tween 20, resuspended 1 ml PBS/0.1% Tween 20 and 2 x 10^5 microparticles analysed using a FACScan flow cytometer (Becton Dickinson). As can be seen from Figure 5, there is no difference in the distribution of fluorescence intensity of beads from all three control reactions (no GST, no CDNB, and no biotin-GSH), where mean fluorescence is ~3. In contrast beads from the enzyme catalysed reaction have a mean fluorescence of 34, over 10 times higher. Indeed, using the gate shown (Fig. 5), 81.1% of beads from the enzyme catalysed reaction (and coated with the biotinylated product) are in the gate whereas in the control reactions no more than 0.06% of beads are in the gate. Hence, beads coated with the product of the GST catalysed reaction can easily be sorted from those which are not.

Example 5.

Glutathione *S*-transferase M2-2 (GST M2-2) will use as a substrate caged-biotinylated-glutathione and the caged-biotinylated product generated can subsequently be uncaged by UV irradiation, captured on avidin-coated beads and detected by flow cytometry

The synthesis of caged-biotin (5) and its derivatives (7) was based on the published protocols (Pirrung & Huang, 1996; Sundberg et al. (1995). However, significant modifications of these protocols were made in several steps of the synthesis as described below.

Biotin methyl ester (3, Biotin-OMe) was prepared essentially as described in Sundberg et al. (1995) (see Fig. 6):

Methylnitropiperonyl alcohol (1, MeNPOH). 3',4-(Methylenedioxy)-6'-nitroacetophenone (Lancaster; 6.2 g., 29.6 mmol) was dissolved in a mixture of THF (100 ml) and ethanol (100 ml). Sodium borohydride (1.12 g., 29.6 mmol) was added and the

solution stirred for 3 hours at room temperature. TLC (on silica coated plates; solvent - 3% methanol in DCM) indicated the full conversion of the starting material (Rf= 0.8) to the alcohol (Rf= 0.6). Hydrochloric acid (1N) was added slowly until the evolution of hydrogen stopped and the solvents evaporated under vacuum. The residual solid was dissolved in DCM (500 ml) and washed with brine (40 ml). The organic phase was dried (over $MgSO_4$) and the solvent removed under vacuum. Recrystallisation from hot DCM and hexane gave 6.1 g. of **1** (a yellow crystalline solid).

O-Methylnitropiperonyl-carbonylimdazole (2, MeNPO-CO-Im).

Methylnitropiperonyl alcohol (1.69 g, 8 mmol) was added (in several portions during 20 minutes) to a solution of carbonyldiimidazole (CDI, 2.6 g, 16 mmol) in DCM (50 ml). The solution was stirred for 3 hrs after which TLC indicated the complete conversion of the alcohol (Rf= 0.6 – 3% methanol in DCM) into product (Rf= 0.45). DCM (100 ml) and water (30 ml) were added and the reaction mixture transferred to a separatory funnel. The mixture was mixed and 1N HCl was added (in 1 ml aliquots) until the pH of the aqueous phase went below 6. The aqueous phase was removed, more water added (30 ml) and acidified to pH 6 while mixed. Finally, the DCM phase was washed with brine, dried (over $MgSO_4$) and the solvent removed under vacuum. The remnant solid was re-crystallised from hot DCM and hexane to give 2.2 g of **2** (a yellow crystalline solid).

N-(O-Methylnitropiperonyl-carbonyl)-Biotin methyl ester (4, MeNPO-CO-Biotin-OMe). Sodium hydride (60% suspension in oil; 100 mg, 2.5 mmol) was added to a stirred suspension of Biotin-OMe (517 mg, 2 mmol) and MeNPO-CO-Im (305 mg, 1 mmol) in anhydrous DCM (10 ml) on ice. The solution was stirred for 30 minutes on ice and 30 minutes at room temperature. TLC indicated the complete disappearance of the MeNPO-CO-Im (Rf= 0.6 – 5% methanol in DCM) and the appearance of the product (Rf= 0.45). Traces of alcohol **1** (Rf=0.7), and a side-product with Rf=0.95 (probably di-MeNPO-carbonate) were also observed (The ratio of product vs. the above side-product varied from one preparation to another; careful drying of the starting materials and performing the reaction on ice gave generally higher yields of the product).

Once the reaction had been completed, DCM was added (100 ml) and the solution extracted three times with 1M NaH_2PO_4. The organic phase was dried ($MgSO_4$) and the solvent removed under vacuum. The remnant syrup was dissolved in hot DCM (ca. 5 ml), hexane (ca. 5 ml) was added to the cloud-point and the solution was allowed to stand at 4°C overnight. This resulted in the precipitation of the excess of the Biotin-OMe as a white crystalline solid (which was washed with ether, dried and used in subsequent reactions). The filtrate was concentrated in vacuum and purified by chromatography on silica (1.5 to 3% methanol in DCM) to give **4** as a yellow foam (with yields up to 385 mg, or 80% based on molar equivalents of **2** as starting material).

N-(O-Methylnitropiperonyl-carbonyl)-Biotin (5, MeNPO-CO-Biotin-OH). MeNPO-CO-Biotin-OMe (940 mg; 1.73 mmol) was dissolved in 25 ml of 0.5N HCl and dioxane (4:6; flashed with argon). The solution was stirred at 44°C for 24 hours under argon. The solvents were reduced under vacuum to ca. 1 ml, water was added (10 ml) and the resulting mixture lyophilised. The resulting solid was dissolved in DCM with 2% methanol (20 ml) and charcoal was added. The mixture was boiled for few minutes and filtered. TLC (10% methanol in DCM) indicated the appearance of the product of the hydrolysis (Rf= 0.2) and about 5% of starting material (MeNPO-CO-Biotin-OMe; Rf= 0.9). The solvents were removed under vacuum to give a yellow solid that was dried under vacuum (860 mg of ca. 95% of **5** plus 5% of **4**). Higher concentrations of HCl (e.g., 1N) and higher temperatures (e.g., reflux with THF as a co-solvent) resulted in complete hydrolysis of the methyl ester. However, significant amount of alcohol **1** and biotin were also observed, indicating the hydrolysis of the carbamate under these conditions. It should also be noted that methyl ester **4**, and in particular, the product of its hydrolysis (**5**) were found to be sensitive to oxidation. Warming or even storing solutions of **5** in the presence of air resulted in browning. Similarly, attempts to purify **5** (or derivatives of, e.g., **7**) by chromatography on silica led to very high losses due to oxidation.

N-(N-(O-Methylnitropiperonyl-carbonyl)-Biotin)-3-aminopropionic acid tert-butyl ester (6, MeNPO-CO-Biotin-β-Ala-OBu^t). MeNPO-CO-Biotin-OH (860 mg containing ~5% of MeNPO-CO-Biotin-Ome; ~1.6 mmol) was dissolved in 20 ml of anhydrous DCM. β-Alanine tert-butyl ester (H-β-Ala-OBu^t) hydrochloride salt (Bachem; 362 mg; 2

mmol), N-hydroxysuccinimide (172 mg; 1.5 mmol) and triethylamine (280 µl; 2 mmol) were added. The stirred solution was cooled on ice and EDCI was added (420 mg; 2.2 mmol). The reaction was stirred for 24 hours at 4°C and 2 hours at room temperature. TLC (5% methanol in DCM) indicated the appearance of the product (Rf= 0.3) and the remaining, unreacted MeNPO-CO-Biotin-OMe (Rf= 0.45). The reaction was diluted with DCM (30 ml) and extracted three times with 1M NaH_2PO_4 and once with saturated $NaHCO_3$. The organic phase was dried (Na_2SO_4) and the solvent removed under vacuum. The remnant syrup was purified by chromatography on silica (3.0-4.5% methanol in DCM) to give 640 mg of **6** (a yellow foam).

N-(N-(O-Methylnitropiperonyl-carbonyl)-Biotin)-3-aminopropionic acid (7, MeNPO-CO-Biotin-β-Ala-OH). Tert-butyl ester **6** (510 mg; 0.84 mmol) was dissolved in 15 ml of 0.5N HCl and dioxane (4:6; flashed with argon). The solution was stirred at 52°C for 24 hours under argon. Water was added (10 ml) and the resulting solution was freeze-dried to give a solid that contained (as judged by TLC) the product of the hydrolysis (**7**) and starting material (**6**; ~ 10%). This mixture was purified by column chromatography on silica (10% methanol in acetone plus 0.1% acetic acid) to give 60mg of **7** (the low yields were primarily the result of oxidation of **7** on the silica).

N-(N-(N-(O-Methylnitropiperonyl-carbonyl)-Biotin)-3-aminopropionyl)-glutathione (8, MeNPO-CO-Biotin-β-Ala-GSH). Carbonyldiimidazole (20 mg, 120 µmol) was added to a solution of MeNPO-CO-Biotin-β-Ala-OH (**7**, 49 mg, 89 µmol) in DMF (1.5 ml). The solution was stirred for 30 minutes at room temperature and was then added, in several aliquots, to a solution of oxidised glutathione (62 mg, 100 µmol) and triethylamine (55 µl, 0.4 mmol), in DMF (2 ml) plus water (0.15 ml), stirred on ice. The solution was stirred on ice for 30 minutes and then at room temperature. Triethylamine was added, until the solution became clear (25 µl), and the reaction was then stirred for another 2 hours at room temperature. DTT was then added (0.25 ml of 1M solution; 0.25 mmol), and the solution was stirred at room temperature for 10 minutes.

The product of the above reaction was purified by reverse-phase HPLC, on an RP-8 preparative column, using a water-acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. The peak corresponding to **8** (retention time = 28.6 minutes) was

collected. The product was then isolated by freeze-drying and purified again on reverse-phase HPLC (using the same column and solvent system). Analysis of the product after the second HPLC purification, using analytical reverse-phase HPLC, indicated a product (>95%) the UV spectrum of which corresponded to **8** (specifically, λ^{max} at 355 nm indicated the presence of the O-methylnitropiperonyl-carbonyl group of the caged-biotin). The concentration of **8** was determined by titrating the free thiol groups (using DTNB, 5,5'-dithio*bis* (2-nitrobenzoic acid), as Hermanson, 1996) derived from the glutathione, and also by absorbance at 355nm (corresponding to the caged-biotin). Both these independent measurements gave the same result within experimental error.

The purified **8** was also found to be a substrate for human M2-2 GST in the electrophilic substitution of CDNB (monitored by the change of absorbance at 340 nm; Habig & Jakoby, 1981) with rates that are about 10 fold slower than those observed with glutathione under similar conditions.

The reduced MeNPO-CO-Biotin-β-Ala-GSH (caged-biotin-βala-GSH) was reacted with either 1-chloro-2,4-dinitrobenzene (CDNB; Sigma) or 4-chloro-3-nitrobenzoate (CNB, Acros). The caged product generated does not bind avidin or streptavidin. However, after photochemical uncaging by ultraviolet radiation the product has a biotin at one end which will bind to avidin or streptavidin-coated microparticles and either a 2,4-dinitrophenol (DNP) or a 3-nitrobenzoate group which can be bound by appropriate anti-DNP or anti-3-nitrobenzoate antibodies (see Figs. 7 & 8)

5 µl (10^8 beads)1.0 µm diameter nonfluorescent neutravidin labelled microspheres (Molecular Probes, F-8777) were spun in a microfuge at 10,000 g for 3 min. and the supernatant removed. The beads were resuspended in 5 µl 0.1 M KH_2PO_4, pH 6.5, 1 mM EDTA, 2 mM dithiothreitol, 10 µM caged-biotin-βala-GSH, and either 500 µM CDNB or 500 µM CNB. The 5 µl reaction mixes contained either 0.75 µg purified recombinant human GST M2-2 or no enzyme.

Reactions were incubated for 30 min (CDNB reactions) or 4 hours (CNB reactions) at 25°C, after which time they were stopped by the addition of 35 µl 0.1 M sodium acetate,

pH 5.0 and transferred to ice. Each reaction was then split into two aliquots of 20 µl each, one of which was placed as a spot on a layer of parafilm on the surface of an ice-cooled aluminium block. This spot was then irradiated for 2 min with a B 100 AP UV lamp (UVP) held at a distance of ~ 6 cm. The other aliquot was left un-irradiated. All samples were then incubated 30 mins. at ambient temperature and then washed three times with 200 µl PBS, 0.1 % Tween 20 in a 0.45 µm MultiScreen-HV filter plate (Millipore, MAHVN4510), thoroughly resuspending between each wash.

Beads were then resuspend in 200 µl PBS, 0.1 % Tween 20 containing 20 ng/µl Alexa-488 labelled rabbit anti-DNP antibody (Dako, #V0401) 20 ng/µl Alexa-488 labelled anti-CNB antisera and incubated for 1 hour at room temperature. The anti-CNB antiserum was elicited in rabbits by immunisation with CNB-CH_2-KLH conjugates prepared by adding aliquots of a 200 mM solution of 4-(bromomethyl)-3-nitrobenzoic acid (CNB-CH_2Br) in DMF to 5 mg/ml solutions of bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) in 50 mM borate pH 8.8 (to give 1.5 to 6 µmole of CNB-CH_2Br per mg protein). The reaction mixtures were stirred for 6 hours at room temperature and temperature, and the resulting protein conjugates were dialysed extensively against phosphate buffer saline (PBS) at 4°C. The level of conjugation (hapten density or Hd) was determined by measuring optical densities of the conjugates at 355nm. These were found to be: 7 to 11 CNB-CH_2 groups per BSA molecule and 9.4 to 24.3 per KLH molecule depending on the amount of CNB-CH_2Br added to the protein samples. The CNB-CH_2-KLH conjugate with Hd of 14.2 was used to immunise rabbits using published protocols (Tawfik et al., 1993; Tawfik et al., 1997) (by Prof. Z Eshhar, Weizmann Institute of Science, Rehovot). Sera were tested by ELISA for binding the conjugate CNB-CH_2-BSA (Hd=11) and to BSA. The first bleed from both immunised rabbits (when diluted 50 fold or more) exhibited the desirable selectivity yielding high signal when incubated with the CNB-CH_2-BSA conjugate and very low background (<5%) with BSA. The anti-CNB serum was purified using a HiTrap Protein A column (Pharmacia). Both anti-CDNB and anti-CNB antibodies were labelled with an Alexa Fluor 488 protein labelling kit (Molecular Probes) according to the manufacturer's instructions.

The beads were washed three times with 200 μl PBS, 0.1 % Tween 20 as above, then resuspended in 1 ml PBS, 0.1 % Tween 20 and 10,000 events analysed using a FACScan flow cytometer (Becton Dickinson).

As can be seen from Fig. 9, the caged-biotin moiety is uncaged on UV irradiation and binds to beads. A 19-fold increase in mean bead fluorescence was observed after GST M2-2 catalysed reaction of caged-biotin-βala-GSH with CDNB even in the absence of UV irradiation. This correlates with the apparent presence of ~4% biotin-βala-GSH in the preparation of caged-biotin-βala-GSH as determined by using fluorimetry to measure the displacement of 2-anilonaphthalene-6-sulphonic acid (2,6-ANS) from avidin (Mock *et al.*, 1985). These results are consistent with the previously observed background immobilisation of caged-biotin to avidin 'in the dark' (i.e., without UV illumination) which was as high as 15% of the signal observed after illumination (Sundberg et al. 1995). The 'dark' signal observed previously was ascribed to either trace contaminants of biotin in the caged-biotin preparation, or to weak interactions between avidin and components of the caged-biotin including the linker (Sundberg et al. 1995). After UV irradiation a large difference in the mean fluorescence of those beads incubated in the presence and absence of GST was observed. The mean bead fluorescence with GST was 84 times and 56 times that observed without GST with CDNB and CNB as substrates respectively (Fig. 9).

Example 6.

Glutathione *S*-transferase M2-2 (GST M2-2) compartmentalised in the aqueous droplets of a water-in-oil emulsion catalyses the reaction of caged-biotinylated-glutathione with 4-chloro-3-nitrobenzoate (CNB). The caged-biotinylated product generated remains compartmentalised and can subsequently be uncaged by UV irradiation in the compartments, captured on an avidin-coated bead in the same compartment and the product-coated beads detected by flow cytometry.

20 μl aliquots (4 x 10^8 beads) of 1.0 μm diameter nonfluorescent neutravidin labelled microspheres (Molecular Probes, F-8777) or 0.93 μm diameter streptavidin-coated polystyrene beads (Bangs Laboratories) were each spun in a microfuge at 2,600 g (6,500 rpm) for 3 min. The supernatant was removed and the beads resuspended, on ice, in 20 μl

0.1 M KH_2PO_4, pH 6.5, 1 mM EDTA, 2 mM dithiothreitol, 50 μM caged-biotin-βala-GSH, containing either 3 μg purified recombinant human GST M2-2 or no enzyme.

Six reaction mixtures were then emulsified essentially as Tawfik & Griffiths (1998):

a) Bangs beads, no GST
b) Bangs beads, plus GST
c) Molecular Probes beads, no GST
d) Molecular probes beads, plus GST
e) Bangs beads, no GST
f) Molecular Probes beads, no GST

The oil phase was freshly prepared by dissolving 4.5% (v/v) Span 80 (Fluka) in mineral oil (Sigma, #M-5904) followed by 0.5% (v/v) Tween 80 (SigmaUltra; #P-8074). Ice-cooled reaction mixtures were added gradually (in 5 aliquots of 4 μl over ~2 minutes) to 0.4 ml of ice-cooled oil-phase in a 5 ml Biofreeze Vial (Costar, #2051) whilst stirring with a magnetic bar (8x3 mm with a pivot ring; Scientific Industries International, Loughborough, UK). Stirring (at 1150 rpm) was continued for an additional 1 minute on ice.

8 μl of emulsion d) was added to 0.4 ml emulsion e), and 8 μl of emulsion b) was added to 0.4 ml emulsion f) (to give 1:50 dilutions) and the emulsion mixtures vortexed for 5 seconds to mix.

Six reaction mixtures were left non-emulsified:

a) Bangs beads, no GST
b) Bangs beads, plus GST
c) Molecular Probes beads, no GST
d) Molecular probes beads, plus GST
e) Bangs beads, no GST
f) Molecular Probes beads, no GST

0.4 μl of d) was added to 20 μl of e), and 0.4 μl b) was added to 20 μl of f) (to give 1:50 dilutions).

Both emulsions and non-emulsified reactions were incubated for 15 min at 25°C. Then 0.8 μl 500 mM CNB (in absolute ethanol) was added to each 0.4 ml emulsion and the emulsion vortexed for 5 seconds (the CNB is transferred through the mineral oil to the aqueous compartments). 5 μl 5 mM CNB (in 0.1 M KH_2PO_4, 1 mM EDTA, pH, 6.5) was added to the non-emulsified reactions.

All reactions were incubating for 4 hours at 25°C

The pH of the aqueous droplets was lowered to quench the GST catalysed reaction by vortexing the emulsions with 200 μl Sigma Mineral Oil for Molecular Biology (M-5904) containing 4.5% Span 80 (Fluka), 0.5% Tween 80 (Sigma Ultra) in Sigma Mineral Oil for Molecular Biology) and 25 mM acetic acid. The non-emulsified reactions were quenched by adding 25 μl 0.5 M acetic acid.

All reactions were transferred to a 24-well flat bottom plate (Corning, #25820) floating on iced water and irradiated for 2 min with a B 100 AP UV lamp (UVP) held at a distance of ~ 6 cm. All samples were then incubated 30 mins. at ambient temperature.

The emulsions were transferred to 1.5 ml microfuge tubes, spun 1 min. 13.5k rpm in a microfuge and the oil phase removed leaving the concentrated (but still intact) emulsion at the bottom of the tube. 200 μl 0.1M Na acetate, pH 5.0 were added and the emulsion broken by extracting 4 times with 1 ml hexane, vortexing between each hexane addition. Residual hexane was removed by spinning for 10 min at ambient temperature under vacuum in a Speedvac (Farmingdale, NY).

All samples were then washed three times with 200 μl PBS, 0.1 % Tween 20 in a 0.45 μm MultiScreen-HV filter plate (Millipore, MAHVN4510), thoroughly resuspending between each wash. Beads were then resuspend in 200 μl PBS, 0.1 % Tween 20. 25μl (~5 x 10^7 beads) were then added to 200 μl PBS, 0.1 % Tween 20 containing 20 ng/μl Alexa-488

labelled anti-DNP antibody or 20 ng/μl Alexa-488 labelled anti-CNB antibody (see Example 5) and incubated for 1 hour at ambient temperature. The beads were washed three times with 200 μl PBS, 0.1 % Tween 20 as above, then resuspended in 1 ml PBS, 0.1 % Tween 20 and 300,000 events analysed using a FACScan flow cytometer (Becton Dickinson).

In the non-emulsified mixtures, where neither GST nor the product of the GST catalysed reaction, (caged-biotin-βAla-NB) were compartmentalised, all beads have a similarly low fluorescence (Fig. 10, Panels B and D). In contrast, in the emulsion mixtures, where both GST and the product of the GST catalysed reaction, (caged-biotin-βAla-NB) were compartmentalised, two populations of beads, one of low and one of higher fluorescence are clearly visible (Fig. 10, Panels C and E). Gating through R1 and R2 enables the Bangs and Molecular Probes beads to be largely separated on the basis of their slightly different light scattering characteristics (Fig 10, Panel A). The ratio of Bangs to Molecular Probes beads passing through R1 is 68%:0.1% and the ratio passing through R2 is 0.08%:87%. Using these gates it is clear that the beads with high fluorescence are those which were compartmentalised with the enzyme GST. Hence, compartmentalisation of beads, enzyme and reaction product was obtained by emulsification and those beads present in compartments which contained enzymes can be distinguished from those which do not by their fluorescence characteristics.

Example 7.

Human GST M2-2 can be transcribed and translated *in vitro* in the aqueous compartments of a water-in oil emulsion and catalyses a reaction which gives rise to a change in the fluorescence properties of co-compartmentalised microspheres.

The gene encoding human glutathione *S*-transferase M2-2 (GST M2-2) is amplified by PCR using oligonucleotides GSTM2-2Fo and GSTM2-2Bc from a human GST M2-2 cDNA clone in pGEM-3Z (Baez et al., 1997). The PCR fragment is cloned into the vector pGEM-4Z (Promega) digested with *Hind*III and *Kpn*I downstream of the *lac* promoter and T7 RNA polymerase promoter. The oligonucleotide GSTM2-2Bc appends the efficient phage T7 gene 10 translational start site upstream of the methyltransferase gene start

codon. DNA sequencing identifies a clone with the correct nucleotide sequence. termed pGEM-hGSTM2-2. The pGEM-hGSTM2-2 plasmid described above is amplified by PCR using primers LMB2 and LMB3 as above to create a 826 base pair PCR fragment (GSTM2-2.LMB2-3) which carries the T7 RNA polymerase promoter, the phage T7 gene 10 translational start site and the GST gene. The PCR fragment is purified directly using Wizard PCR Preps (Promega).

60 μl aliquots (1.2 x 10^9 beads) of 1.0 μm diameter nonfluorescent neutravidin labelled microspheres (Molecular Probes, F-8777) were spun in a microfuge at 10,000 g for 3 min. The supernatant was removed and the beads resuspended ,on ice, in 60 μl of a prokaryotic *in vitro* coupled transcription/translation system designed for linear templates (Lesley et al., 1991). A commercial preparation of this system is used (*E. coli* S30 Extract System for Linear Templates; Promega) supplemented with 12.5 mM acetic acid (to lower the pH to ~7.0), T7 RNA polymerase (2,000 units), 12.5 μg/ml λ DNA-*Hind*III digest (New England Biolabs), 50 μM caged-biotin-βala-GSH, and, optionally, 5 nM GSTM2-2.LMB2-3 DNA or 5.0 μg of purified recombinant human GST M2-2 *per* 50 μl (or neither).

A 5 μl aliquot was removed from each reaction mixture and left non-emulsified. 50 μl of the remaining reaction mixture was emulsified essentially as Tawfik & Griffiths (1998).

The oil phase was freshly prepared by dissolving 4.5% (v/v) Span 80 (Fluka) in mineral oil (Sigma, #M-5904) followed by 0.5% (v/v) Tween 80 (SigmaUltra; #P-8074). Ice-cooled reaction mixtures were added gradually (in 5 aliquots of 10 μl over ~2 minutes) to 1.0 ml of ice-cooled oil-phase in a 5 ml Biofreeze Vial (Costar, #2051) whilst stirring with a magnetic bar (8x3 mm with a pivot ring; Scientific Industries International, Loughborough, UK). Stirring (at 1150 rpm) was continued for an additional 1 minute on ice.

Both emulsions and non-emulsified reactions were incubated for 45 min at 25°C to allow translation to proceed. Then 5 μl 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) (in absolute ethanol) was added to each 1.0 ml emulsion and the emulsion vortexed for 5

seconds (the CDNB is transferred through the mineral oil to the aqueous compartments). 1.0 µl 2.5 mM CDNB (in water) was added to the non-emulsified reactions. CDNB inhibits *in vitro* translation and adding it in this way, after translation is completed, maximises the yield of GST.

All reactions were incubating for 30 mins at 25°C. The pH of the aqueous droplets was then lowered to quench the reaction by vortexing the emulsions with 500 µl Sigma Mineral Oil for Molecular Biology (M-5904) containing 4.5% Span 80 (Fluka), 0.5% Tween 80 (Sigma Ultra) in Sigma Mineral Oil for Molecular Biology) and 25 mM acetic acid. The non-emulsified reactions were quenched by adding 5 µl 0.5 M acetic acid and 20 µl 0.1M Na acetate, pH 5.0.

All reactions were transferred to a 24-well flat bottom plate (Corning, #25820) floating on iced water and irradiated for 2 min with a B 100 AP UV lamp (UVP) held at a distance of ~ 6 cm. All samples were then incubated 30 mins. at ambient temperature.

The emulsions were transferred to 1.5 ml microfuge tubes, spun 1 min. 13.5k rpm in a microfuge and the oil phase removed leaving the concentrated (but still intact) emulsion at the bottom of the tube. 200 µl 0.1M Na acetate, pH 5.0 were added and the emulsion broken by extracting 4 times with 1 ml hexane, vortexing between each hexane addition. Residual hexane was removed by spinning for 10 min at ambient temperature under vacuum in a Speedvac (Farmingdale, NY).

Approximately 5 x 10^7 beads from the broken emulsions and the non-emulsified reactions were then washed three times with 200 µl PBS, 0.1 % Tween 20 in a 0.45 µm MultiScreen-HV filter plate (Millipore, MAHVN4510), thoroughly resuspending between each wash. Beads were then resuspend 200 µl PBS, 0.1 % Tween 20 containing 10 ng/µl Alexa-488 labelled anti-DNP antibody (see Example 5) and incubated for 1 hour at ambient temperature. The beads were washed three times with 200 µl PBS, 0.1 % Tween 20 as above, then resuspended in 1 ml PBS, 0.1 % Tween 20 and 10,000 events analysed using a FACScan flow cytometer (Becton Dickinson).

As can be seen from Fig. 11, both in emulsified and non-emulsified reactions, the reaction catalysed by *in vitro* translated GST M2-2 results in an in beads with higher fluorescence than when no enzyme was present. This difference in fluorescence would, however, not be sufficient for efficient fluorescence activated sorting (FACS). However, beads from both emulsified and non-emulsified reactions containing 5.0 μg of purified recombinant GST M2-2 *per* 50 μl were even more fluorescent than those containing *in vitro* translated GST M2-2 enabling efficient enrichment of these beads by FACS from those incubated in the absence of GST. This simulates the situation where a mutant GST of higher activity than wild-type is translated *in vitro*.

Example 8.

Genes attached to microbeads are expressed *in vitro* and the resulting gene-product (an enzyme) binds to the microbeads whilst retaining catalytic activity.

One format for the selection of genetic elements is where the genetic element comprises a gene linked to a microbead, which is translated in a microcapsule, and the translated gene-product is coupled back onto the microbead within the microcapsule. Thus, compartmentalisation leads to the formation of complexes of gene-products (e.g., proteins or enzymes) attached to the gene encoding them. These complexes could be subsequently selected for binding a ligand (see Example 12), or for enzymatic activity via a second compartmentalised reaction.

Here it is shown, that an enzyme (phosphotriesterase or PTE) can be transcribed and translated *in vitro* from genes attached to microbeads and the translated enzyme is bound back the microbeads. We also show that the translated enzyme can be modified, assembled or complemented with a cofactor whilst it is bound on the beads - in this example, metal ions are added to the apo-enzyme to give an active metalloenzyme. Moreover, we show here that the catalytic activity of the enzyme is retained whilst it is bound to the microbead together with the gene that encodes it.

The *opd* gene encoding a phosphotriesterase (PTE; also known as paraoxon hydrolase; Mulbry & Karns, 1989) is amplified from *Flavobacterium* sp. strain ATCC 27551 by PCR using a forward primer that appends stop codons and an *EcoR*I site (OPD-Fo; see

Table 1), and a back primer that appends the phage T7 gene 10 transitional site (RBS) and a *Hind*III cloning site (OPD-Bc). This DNA is cloned into pGEM-4Z using the *Hind*III and the *EcoR*I sites downstream of the T7 RNA polymerise promoter. DNA sequencing identifies a clone which has the correct nucleotide sequence. Bacteria (*E.* coli, TG1) transformed with this clone (Gem-OPD) are found to overexpress active PTE when grown in the presence of cobalt chloride and induced with IPTG (Omburo *et al.*, 1992).

The OPD gene is also cloned with a Flag™ peptide (Met-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; Sigma-Aldrich) appended to its N-terminus. The OPD gene is amplified *Flavobacterium* sp. strain ATCC 27551 by PCR using a forward primer (N-Flag-OPD-Fo) that appends stop codons and a *Kpn*I site, and a back primer (N-Flag-OPD-Bc) appending an *Nco*I site, a Flag peptide and a short linker between the Flag peptide and the OPD reading frame. The resulting DNA fragment is cloned into plasmid pGEM-4Z^{NcoI} (using the *Kpn*I and *Nco*I sites). pGEM-4Z^{NcoI} is a modification of p-GEM-4Z into which, the phage T7 gene 10 transitional site (RBS) and an ATG start codon are appended downstream to the T7 RNA polymerise promoter, to create an *Nco*I site that allows cloning of reading frames in the context of the RBS and ATG codon. The sequence of the section incorporated into pGEM-4Z (between the *Hind*III and the *Kpn*I sites downstream to the T7 RNA polymerise promoter), to give pGEM-4Z^{NcoI}, is indicated in Scheme I.

The rest of pGEM-4Z, including the *Kpn*I and *EcoR*I cloning sites, remained intact.

----- 5'-<u>AAGCTT</u>AATAATTTTGTTTAACTTTAAGAAGGAGATATAG<u>*CCATGG*</u>...

pGEM-4Z - *Hind*III site   appended RBS, ATG and *Nco*I cloning site

....<u>GGTACC</u>-3'--------

*Kpn*I site of pGEM-4Z

Scheme I

DNA sequencing identifies a clone that has the correct nucleotide sequence. Bacteria transformed with this clone (Gem-N-Flag-OPD) are found to over-express an active PTE when grown in the presence of Cobalt Chloride and induced with IPTG.

The gem-OPD and gem-N-Flag-OPD plastids described above are amplified by PCR, using primers LMB2-biotin and LMB3, to create DNA fragments (OPD.LMB3-2biotin and N-Flag-OPD.LMB3-2biotin, respectively) that carry the T7 RNA polymerise promoter, the phage T7 gene 10 transitional start site and the OPD or the N-Flag-OPD genes and are labelled with biotin at the 3' end. The PCR fragments are purified directly using Wizard PCR Preps (Promega).

Aliquots of a suspension of 0.95 μm non-fluorescent streptavidin labelled microspheres (Bangs, ~2 x 10^7 beads per μl suspension) are spun in a microfuge at 10,000 g (13.500 rpm) for 3 min. The supernatant is removed and the beads resuspended in TNT buffer (0.1M Tris 7.5, 0.15M NaCl, 0.05% Tween-20). An antibody that is capable of binding amino-termini Flag peptides and is labelled by biotinylation (BioM5, a biotin-labelled anti-Flag antibody; Sigma) is added to the bead suspensions to an average of 4 x 10^4 antibody molecules per bead. The resulting mixture is incubated for several hours with occasional mixing. The beads are rinsed twice by spinning down and resuspending them in TNT buffer. Biotinylated DNA fragments (fragments OPD.LMB3-2biotin, N-Flag-OPD.LMB3-2biotin, or fragments that carry the T7 RNA polymerise promoter, the phage T7 gene 10 transitional start site and a gene encoding a different enzyme that is also tagged with N-Flag peptide, e.g., methyltransferase Hae*III* – N-Flag-M.Hae*III*.LMB3-2biotin) are added to a suspension of antibody-coated beads and the mixture is incubated overnight at 4°C. The beads are rinsed 3 times by spinning down and resuspending them in TNT buffer.

50 μl aliquots of the above suspension of beads (~10^9 beads) are spun in a microfuge at 10,000 g for 3 min. The supernatant is removed and the beads gently resuspended, on ice, in 50 μl of a prokaryotic *in vitro* coupled transcription/translation system designed for linear templates (Lesley et al., 1991). A commercial preparation of this system is used (*E. coli* S30 Extract System for Linear Templates; Promega) supplemented with T7 RNA polymerise (2,000 units). The reactions are incubated at 25°C for 1.5 hours and spun in a microfuge at 10,000 g for 3 min. The supernatant is removed and the beads resuspended in 100 μl of 50 mM Tris, 10 mM of Potassium Carbonate, pH 8.0. An aqueous solution of Cobalt Chloride is added to a concentration of 1 mM and the reactions incubated for

several hours at room temperature (or overnight at 4°C). The beads are rinsed 4 times by spinning down and resuspending them in TNT buffer.

Aliquots of the above beads are added to a solution of 0.25 mM Paraoxon in 50 mM Tris pH 8.3. The beads are incubated at 25°C with occasional stirring for different periods of time. The beads are spun in a microfuge at 10,000 g for 3 min, the supernatant is removed and its optical density measured at 405nm. A significant change in optical density, relative to the optical density observed under the same conditions in the absence of beads or phosphotriesterase, is not observed when beads to which biotinylated DNA fragments OPD.LMB3-2biotin or N-Flag-M.Hae*III*.LMB3-2biotin are attached (and are subsequently reacted as described above) are incubated with Paraoxon. However, a significant change in optical density at 405nm is observed when beads to which biotinylated DNA fragments N-Flag-OPD.LMB3-2biotin are attached (and are subsequently reacted as described above) are incubated with Paraoxon. For example, when biotinylated DNA fragments N-Flag-OPD.LMB3-2biotin are added at a concentration of 1 nM (to a 50 μl suspension of beads (~10^9 beads) that is then resuspended in 50 μl *in vitro* transcription/translation), and reacted as described above, the change in optical density observed after 3 hours corresponds to more than 50% hydrolysis of Paraoxon (at 0.25 mM in a 50 μl reaction volume). Thus, microbeads carrying a gene encoding a protein with the desired catalytic activity (phosphotriesterase in the above example) can be clearly distinguished from microbeads carrying genes that do not encode a protein with the desired catalytic activity (methyltransferase *Hae*III in the above example). Moreover, almost no change in optical density at 405nm is observed when biotinylated DNA fragments N-Flag-OPD.LMB3-2biotin are attached to beads and reacted as described above, except that Cobalt Chloride is not added to the resuspended beads after transcription/translation.

These results show that an enzyme (phosphotriesterase) can be transcribed and translated *in vitro* from genes that encode this enzyme and are attached to microbeads. When the genes encode a tag – an N-terminus Flag peptide in the above example - the translated enzyme binds back to the microbeads to which the genes are attached. If necessary, the translated enzyme can be then modified whilst it remains attached to the microbeads

(together with the gene that encodes it) – in this example, Cobalt ions are added to give a reactive metallo-enzyme. These result also indicate that the enzyme is catalytically active whilst it is bound to microbeads together with the gene that encodes it.

Example 9.

An enzyme catalyses a reaction with a caged-biotinylated substrate, and the caged-biotinylated product generated is uncaged by UV irradiation and captured on streptavidin-coated microbeads. Subsequently these beads are detected by flow-cytometry.

One format for the selection of genetic elements is where the genetic element comprises a gene linked to a microbead, which is translated in a microcapsule, and the translated gene-product is coupled back onto the microbead within the microcapsule. Thus, compartmentalisation leads to the formation of complexes of gene-products (e.g., proteins or enzymes) attached to the gene encoding them. These complexes could be subsequently selected for binding a ligand (see Example 12), or for enzymatic activity via a second compartmentalised reaction.

However, for such complexes to be selected for catalytic activity, a soluble substrate should be available for the immobilised enzyme, and, once the catalytic reaction had been completed, the product of the enzymatic activity that is being selected for should become attached to the gene encoding this enzyme. The resulting complexes could be then sorted or selected by virtue of the product being linked to them, for example by using a fluorescently-labelled antibody that recognises the product. In other compartments, containing complexes of genes and gene-products that do not encode proteins with the desired enzymatic activity, the unreacted substrate should become linked to the gene. These complexes will not be labelled with the product and will therefore be discarded.
Here it is shown that an enzyme (phosphotriesterase or PTE) can react with a caged-biotinylated substrate in the presence of streptavidin-coated beads. The caged-biotinylated product generated can then be uncaged by UV irradiation and captured on avidin-coated beads. Subsequently, these beads are detected by flow cytometry and are clearly

distinguished from beads incubated with a caged-biotinylated substrate in the presence of other enzymes or proteins that do not exhibit phosphotriesterase activity.

A caged-biotinylated substrate for PTE (EtNP-Bz-Glu-cagedBiotin; Fig. 12) is synthesised as follows:

Boc-5-aminopentanol: Di-tert-butyl dicarbonate (20.8 g; 0.095 mol) is added to stirred solution of 5-aminopentanol (10.37 g; 0.1 mol) in dicholoromethane (DCM) (200 ml) on ice. Following addition, the solution becomes turbid and a syrup separates. Triethylamine is added (13.8 ml; 0.1 mol) drop-wise, and the resulting solution is stirred for 10 minutes on ice and then overnight at room temperature. The solvents are removed under vacuum, the resulting syrup is dissolved in ethyl acetate (500 ml), extracted 3 times with 1M Na_2H-PO_4 (pH 4), once with saturated $NaHCO_3$, and finally with brine, and then dried over $MgSO_4$. The solvents are removed under vacuum and the resulting syrup (after extensive drying under vacuum in the presence of potassium hydroxide), comprised primarily of Boc-5-aminopentanol, is used without further purification.

(**11**) Triethylamine (3 ml; 22 mmol) is added drop-wise to a stirred solution of p-nitrophenyl phoshphodichloridate (5.15 g; 20 mmol) and ethanol (1.15 ml, 20 mmol) cooled on dry-ice in acetone, with in 30 minutes. The solution is allowed to slowly warm up to room temperature and is stirred for an additional 90 minutes. A solution of Boc-5-aminopentanol (4.3 g; ca. 20 mmol) and trietheylamine (3 ml; 22 mmol) in DCM (20 ml) is then added drop-wise. The reaction is allowed to stir at room temperature for 10 minutes, 1*H*-tetrazole is added (0.35 g; 5 mmol) and the reaction stirred for another 2 hours. DCM is added (100 ml) and the solution extracted 3 times with 1M Na_2HPO_4 (pH 4), saturated $NaHCO_3$, and finally with brine, and then dried over $MgSO_4$. The solvents are removed under vacuum to give a syrup that is purified by column chromatography on silica (solvent: 1% to 2% methanol in DCM) to give 3.52 g of **11** (a syrup).

4-N-Boc-aminomethylbenzoic acid N-hydroxy succinimide ester: Dicyclohecyldicarbodiimide (DCC; 5.15 g; 25 mmol) is added to a stirred suspension of 4-N-Boc-aminomethylbenzoic acid (Tiger, Monmouth NJ; 5.2 g; 25 mmol) and N-

hydroxy succinimide (2.88 g; 25 mmol) in DCM (200 ml) plus acetonitrile (20 ml). The reaction is stirred overnight at 4°C and then 3 hours at room temperature. The dicyclohecyl urea precipitate is removed by filtration, and the filtrate concentrated under vacuum to give a syrup. The syrup is dissolved in chloroform and DCM and treated with activated charcoal. Addition of ether gives a white crystalline solid. Recrystallisation from DCM and petroleum ether gives 6.2 g of the N-hydroxy succinimide ester of 4-N-Boc-aminomethylbenzoic acid.

(12) Trifluoroacetic acid (TFA; 4 ml) is added to a solution of **11** (900 mg; 2.07 mmol) in DCM (5 ml). The solution is left at room temperature for 45 minutes and the solvents are removed under vacuum. The residual syrup is triturated by dissolving it DCM and methanol and adding ether. The resulting **12** (as TFA salt; syrup) is dried over vacuum in the presence of potassium hydroxide, and then reacted immediately without further purification (see below).

(13) 4-N-Boc-aminomethylbenzoic acid N-hydroxy succinimide ester (670 mg; 2.2 mmol) and triethylamine (0.345 ml; 2.5 mmol) are added to **12** (see above) in DCM (15 ml). The solution is stirred for 30 minutes, triethylamine (0.1 ml; 0.72 mmol) is added, and the solution stirred for additional 3 hours. DCM is added (20 ml), and the solution extracted twice with 1M Na_2HPO_4 (pH 4), once with saturated $NaHCO_3$, and finally with brine, and then dried over $MgSO_4$. The solvents are removed under vacuum to give a syrup that is purified by column chromatography on silica (solvent: 5% methanol in DCM) to give 0.86 g of **13** (a syrup).

(14) 0.84 g **13** of 14 (1.6 mmol) is treated with TFA as described above to give **14** (as TFA salt; syrup) which is reacted immediately as described below.

(15) Boc-Glu(OSu)-OBu^t (Bachem; 641 mg; 1.6 mmol) and triethylamine (0.235 ml; 1.7 mmol) are added to **14** (see above) in DCM (15 ml). The solution is stirred for 1 hour, triethylamine (60 µL; 0.43 mmol) is added, and the solution stirred for 1 hour. DCM is added (20 ml), and the solution extracted twice with 1M Na_2HPO_4 (pH 4), once with saturated $NaHCO_3$, and finally with brine, and then dried over $MgSO_4$. The solvents are

removed under vacuum to give a syrup that is purified by column chromatography on silica (solvent: 7% methanol in DCM) to give 0.8 g of **15** (a white crystalline solid).

EtNP-Bz-Glu (16) 0.4 g of **15** (0.56 mmol) are dissolved in DCM (5 ml) and TFA (5 ml). The solution is stirred for 1 hour at room temperature, and the solvents are removed under vacuum. The residual syrup is crystallised by dissolving it methanol and adding ether. Recrystallisation (in methanol and ether) gives 200 mg of **16** (as TFA salt; white solid).

EtNP-Bz-Glu-cagedBiotin (17) Carbonyldiimidazole (6 mg, 37.5 μmol) is added to a solution of MeNPO-CO-Biotin-OH (**5**, 17 mg, 35 μmol) in DMF (1 ml). The solution is stirred for 60 minutes at room temperature and added to **16** (20 mg, 30 μmol). Triethylamine (5.5 μl, 40 μmol), DMF (1 ml) and water (0.5 ml) are added to the stirred reaction mixture until it became clear. The solution is stirred for 2 hours at room temperature and stored at –20°C.

The product of the above reaction is purified by reverse-phase HPLC on a C8 preparative column using a water-acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. The peak corresponding to **17** (retention time = 23.1 minutes) is collected. The product is isolated by freeze-drying as a yellow solid. Analysis of the product after the HPLC purification using analytical reverse-phase HPLC indicated a major product (>80%), the UV spectrum of which corresponded to **17**. Specifically, λ^{max} at 355nm indicates the presence of the O-methylnitropiperonyl-carbonyl group of the caged-biotin (Pirrung & Huang, 1996), and a 'shoulder' at 277nm, absent in caged-biotin, indicates the presence of the p-nitrophenyl phosphate ester of **17**. The concentration of **17** is verified by hydrolysing the p-nitrophenyl phosphate ester in 0.1M potassium hydroxide and determining the amount of p-nitrophenol released (optical density at 405nm).

The purified **17** is also found to be a substrate for PTE leading to the release of p-nitrophenol (Fig. 13; monitored by the change in optical density at 405nm) with rates that are only about 6 fold slower than those observed with Paraoxon. Notably, unlike the base-catalysed hydrolysis of **17** which proceeds to completion (and the PTE-catalysed hydrolysis of Paraoxon), the PTE-catalysed hydrolysis of **17** proceeds with significant

rates only until half of the substrate has been hydrolysed. The second half of the substrate could also be hydrolysed, but only in the presence of much higher quantities of PTE and after long incubations (several hours to overnight). This is probably due to the fact that there **17** is comprised of two diastereomers (corresponding to two enantiomers with regard to the chiral phosphotriester), only one of which is an effective substrate for the enzyme. Indeed, stereoselectivity was previously observed with PTE and other chiral phosphotriesters (Hong & Raushel, 1999).

Antibodies are generated that would recognise ethyl-phosphodiesters that are the products of hydrolysis of the corresponding p-nitrophenyl phosphotriesters. To this end, a suitable ethylphosphodiester derivative is synthesised and conjugated to carrier proteins as described below (Fig. 14).

EtNPBG (18) (Glutaric anhydride (180 mg; 1.6 mmol) and triethylamine (0.22 ml; 1.6 mmol) are added to **12** (prepared by de-protection of 1.6 mmol of **11**, as described above) in DCM (15 ml). The solution is stirred for 20 minutes, triethylamine (0.12 ml; 0.85 mmol) is added, and the solution stirred for an additional 1 hour. DCM is added (20 ml), and the solution extracted twice with 1M Na_2HPO_4 (pH 4) and then dried over $MgSO_4$. The solvents are removed under vacuum to give a syrup that is purified by column chromatography on silica (solvent: 12.5% methanol in DCM *plus* 0.1% acetic acid) to give 445 mg of **18** (a syrup).

Substrate conjugates EtNPBG-KLH and EtNPBG-KLH. Carbonyldiimidazole (CDI; 32 mg, 200 μmol) is added to a solution of **18** (60 mg, 134 μmol) in DMF (1 ml). The solution is stirred for 60 minutes at room temperature. Aliquots of the activated **18** are then added to 5 mg/ml solutions of bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) in 0.1M phosphate pH 8.0 (at 0.5 to 4 μmole of **18** per mg protein). The reactions are stirred for 1 hour at room temperature, and the resulting protein conjugates are dialysed extensively against phosphate buffer saline (PBS) at 4°C. The level of conjugation (hapten density or Hd) is determined by hydrolysing a sample of the dialysed conjugates in 0.1M potassium hydroxide and monitoring the amount of released p-nitrophenol (at 405nm). These are found to be: 8.5 to 24 EtNPBG molecules per BSA

molecule and 14 to 63 per KLH molecule depending on the amount of activated **18** added to the protein samples.

Product conjugates EtBG-KLH and EtBG-KLH. The EtNPBG-KLH and EtNPBG-KLH conjugates described above are dialysed against 0.1M carbonate pH 11.8 for 44 hours at room temperature, and then extensively against PBS (at 4°C).

Anti-EtBG antibodies were elicited in rabbits by immunisation with EtBG-KLH (Hd=14) using published protocols (Tawfik *et al.*, 1993; Tawfik *et al.*, 1997) (gift of Prof. Z Eshhar, Weizmann Institute of Science, Rehovot). Sera are tested by ELISA for binding to both the substrate conjugate EtNPBG-BSA (Hd=8.5) and the corresponding product conjugate (EtBG-BSA; Hd=8.5). The first bleed from one of the immunised rabbits (when diluted 500 fold or more) exhibits the desirable selectivity, yielding high signal when incubated with the product conjugate and a low background (<20%) with the substrate conjugate. Diluting the sera in COVAp buffer (2M NaCl, 10 g, 1 $MgSO_4 \cdot 7H_2O$, 0.04% Tween-20, 10 mM phosphate, 0.1 mM p-nitrophenol, pH 6.5) further increases selectivity, with background levels going below 5%. The anti-EtBG serum is purified using a HiTrap Protein A column (Pharmacia). The purified rabbit antibodies are labelled with an Alexa Fluor 488 protein labelling kit (Molecular Probes) according to the manufacturer's instructions.

10 μl (~2 x10^8 beads) of 0.95 μm streptavidin-coated microbeads (Bangs, ~2 x 10^7 beads per μl suspension) are spun in a microfuge at 10,000 g for 3 min. and the supernatant removed. The beads are resuspended in 10 μl of 50 mM Tris pH 8.3 containing EtNP-Bz-Glu-cagedBiotin (17) to give a final concentration of 10 μM, 20 μM or 30 μM. PTE is expressed *in vitro* by transcription/translation of OPD.LMB3-2biotin DNA fragments (at 5 nM). A commercial preparation is used (*E. coli* S30 Extract System for Linear Templates; Promega) supplemented with T7 RNA polymerise (2,000 units) and the reactions are incubated at 25°C for 1.5 hours. The PTE is then assembled by the addition of Potassium Carbonate (10 mM) and Cobalt Chloride (1mM) in Tris buffer (10 mM pH 8.0) and incubating for overnight at 4°C. Another enzyme, that does not exhibit phosphotriesterase activity, methyltransferase Hae*III*, is also expressed *in vitro* by transcription/translation from M.Hae*III*.LMB3-2biotin DNA fragments (at 5 nM), and

then treated with carbonate and cobalt as with the PTE. 5 µl aliquot of the above reaction mixtures are added to the bead suspensions and the reactions are incubated for 1 hour at 25°C in the dark. The reaction is stopped by the addition of 15 µl 0.1 M sodium acetate, pH 5.0 and transferred to ice. Each reaction is then split into two aliquots of 15 µl each, one of which is placed as a spot on a layer of parafilm on the surface of an ice-cooled aluminium block. This aliquot is then irradiated for 2 min with a B 100 AP UV lamp (UVP) held at a distance of ~6 cm. The other aliquot is left in the dark. All bead samples are then incubated for 30 minutes at ambient temperature and washed three times with 200 µl PBS, 0.1 % Tween 20 in a 0.45 µm MultiScreen-HV filter plate (Millipore, MAHVN4510), thoroughly resuspending between each wash. Beads (~2 x 10^7) are then resuspended in 200 µl COVAp containing 100 ng/µl Alexa-488 labelled rabbit anti-EtBG antibodies and incubated for 1 hour at room temperature and then 1 hour at 4°C. The beads were washed three times with 200 µl PBS, 0.1 % Tween 20 as above, then resuspended in 1 ml PBS, 0.1 % Tween 20 and 10,000 events analysed using a FACScan flow-cytometer (Becton Dickinson).

As can be seen in Fig. 15, up to 20-fold increase in mean bead fluorescence is observed following the PTE catalysed hydrolysis of EtNP-Bz-Glu-cagedBiotin in the presence of streptavidin-coated beads and after UV irradiation. This is increase is observed relative to beads treated essentially the same but in the presence of another enzyme (M.*Hae*III), with no phosphotriesterase activity. Notably, the differences in fluorescence signal are observed when both the PTE and the M.*Hae*III, are expressed *in vitro* from the corresponding genes and are added together with the entire content of the *in vitro* transcription/translation reaction mixture.

At high substrate concentrations the observed mean fluorescence is lower than observed at 20 µM. In addition, at substrate concentrations above 20 µM, there is essentially no difference in the fluorescence signal between reactions kept in the dark and those UV irradiated (data not shown). Since the beads, under the reaction conditions described above, start to exhibit saturation of binding signal at concentrations above 10 µM (of product as detected by the subsequent addition of fluorescently-labelled anti-EtBG antibodies), these results may be explained by the presence of a contamination of ETNP-

Bz-Glu-Biotin in the preparation of EtNP-Bz-Glu-cagedBiotin. These results are also consistent with the previously observed background immobilisation of caged-biotin to avidin 'in the dark' (i.e., without UV illumination) which was as high as 15% of the signal observed after illumination (Sundberg et al. 1995). The 'dark' signal observed previously was ascribed to either trace contaminants of biotin in the caged-biotin preparation, or to weak interactions between avidin and components of the caged-biotin including the linker (Sundberg et al. 1995). Both mechanisms may account for the fact that at high concentrations of caged-biotinylated substrate (and above the binding capacity of the beads), the 'dark' signal becomes significant. Nevertheless, at substrate concentrations of 20 μM, or lower, the 'dark' signal constitutes only 25%, or even less than 10% (e.g., at 10 μM EtNP-Bz-Glu-cagedBiotin) of the illuminated signal. This indicates that most of the PTE-catalysed hydrolysis of EtNP-Bz-Glu-cagedBiotin takes place whilst the substrate is in solution and not attached to the beads, and that the resulting product (Et-Bz-Glu-cagedBiotin), after illumination with UV light, is un-caged and becomes immobilised onto the microbeads.

Example 10.

Genes attached to beads are expressed *in vitro* and the resulting gene-products (enzymes) become immobilised to the microbeads whilst retaining catalytic activity. The immobilised enzyme catalyses a reaction with a caged-biotinylated substrate, and the resulting caged-biotinylated product is subsequently uncaged by UV irradiation and becomes attached to these beads together with the gene encoding the enzyme that led to its formation. Subsequently, these beads are detected by flow-cytometry.

One format for the selection of genetic elements is where the genetic element comprises a gene linked to a microbead, which is translated in a microcapsule, and the translated gene-product is coupled back onto the microbead within the microcapsule. Thus, compartmentalisation leads to the formation of complexes of gene-products (e.g., proteins or enzymes) attached to the gene encoding them. These complexes could be subsequently selected for binding a ligand (see Example 12), or for enzymatic activity via a second compartmentalised reaction.

For such complexes to be selected for catalytic activity, a soluble substrate should be available for the immobilised enzyme, and, once the catalytic reaction had been completed, the product of the enzymatic activity that is being selected for should become attached to the gene encoding this enzyme. The resulting complexes could be then sorted or selected by virtue of the product being linked to them, for example by using a fluorescently-labelled antibody that recognises the product. In other compartments, containing complexes of genes and gene-products that do not exhibit the desired enzymatic activity, the unreacted substrate would become linked to the gene. These complexes will not be labelled with the product and will therefore be discarded.

Here it is shown that an enzyme (phosphotriesterase or PTE) can be transcribed and translated *in vitro* from genes attached to microbeads and the translated enzyme is bound back to the microbeads. The translated enzyme can be then modified to incorporate the active-site Cobalt, and its catalytic activity is retained whilst it is bound to the microbead together the gene that encodes it. The immobilised PTE subsequently reacts with a caged-biotinylated substrate, and the caged-biotinylated product generated is uncaged by UV irradiation and captured onto the same avidin-coated beads to which the gene encoding the PTE is attached. Subsequently these beads are detected by flow-cytometry and are clearly distinguished from beads carrying a gene encoding a protein that does not exhibit phosphotriesterase activity.

Aliquots of a suspension of 0.95 μm streptavidin-coated microspheres (Bangs, ~2 x 10^7 beads per μl suspension) are spun in a microfuge at 10,000 g for 3 min. The supernatant is removed and the beads resuspended in TNT buffer (0.1M Tris 7.5, 0.15M NaCl, 0.05% Tween-20). An antibody, capable of binding the Flag peptide and biotinylated (BioM5, a biotin-labelled anti-Flag antibody; Sigma) is added to the bead suspensions to give an average of 10^4 antibody molecules per bead and the mixture is incubated for several hours. The beads are rinsed by spinning down and resuspending them in TNT buffer to the original volume. Biotinylated DNA fragments N-Flag-OPD.LMB3-2biotin, or fragments that carry the T7 RNA polymerise promoter, the phase T7 gene 10 transitional start site and a gene encoding a different enzyme (also tagged with N-Flag peptide), e.g., methyltransferase Hae*III* – N-Flag-M.Hae*III*.LMB3-2biotin) are added to the suspension

of antibody-coated beads at 1.6 nM concentration and the mixture is incubated overnight at 4°C. The beads are rinsed 3 times by spinning down and resuspending them in TNT buffer.

50 μl aliquots of the above suspension of beads (~10^9 beads) are spun in a microfuge at 10,000 g for 3 min. The supernatant is removed and the beads gently resuspended, on ice, in 50 μl of a prokaryotic *in vitro* coupled transcription/translation system designed for linear templates (Lesley et al., 1991). A commercial preparation of this system is used (*E. coli* S30 Extract System for Linear Templates; Promega) supplemented with T7 RNA polymerise (2,000 units). The reactions are incubated at 25°C for 1.5 hours and spun in a microfuge at 10,000 g for 3 min. The supernatant is removed and the beads resuspended in 100 μl of 50 mM Tris, 10 mM of potassium carbonate, pH 8.0. An aqueous solution of Cobalt Chloride is added to a concentration of 1 mM and the reactions incubated for 2 hours at room temperature. The beads are rinsed 4 times by spinning down and resuspending them in TNT buffer. Finally, beads are resuspended in TNT buffer to the original volume.

Aliquots of the above beads are added to solutions of 0.25 mM Paraoxon in 50 mM Tris pH 8.3. The beads are incubated at 25°C with occasional stirring for different periods of time. The beads are spun in a microfuge at 10,000 g for 3 min, the supernatant is removed and its optical density measured at 405nm. A significant change in optical density at 405nm is observed when beads to which biotinylated DNA fragments N-Flag-OPD.LMB3-2biotin are attached (and are subsequently reacted as described above) in contrast to reactions conducted under the same conditions but in the absence of beads or phosphotriesterase, or with beads to which N-Flag-M.Hae*III*.LMB3-2biotin DNA fragments are attached and are subsequently reacted as described above.

Next, 10 μl (~2 x10^8 beads) of the above beads are spun in a microfuge at 10,000 g for 3 min. and the supernatant removed. The beads are resuspended in 10 μl of 12.5 or 25 μM EtNP-Bz-Glu-cagedBiotin in 50 mM Tris pH 8.3. The bead suspensions are incubated for 1.5 hour at 25°C in the dark. The reaction is stopped by the addition of 10 μl 0.1 M sodium acetate, pH 5.0 and transferred to ice and irradiated for 2 min with a B 100 AP

UV lamp (UVP) held at a distance of ~ 6 cm. All bead samples are then incubated for 30 minutes at ambient temperature and then washed three times with 200 µl PBS, 0.1 % Tween 20 in a 0.45 µm MultiScreen-HV filter plate (Millipore, MAHVN4510), thoroughly resuspending between each wash. Beads (~7 x 10^7) are then resuspend in 125 µl of a rabbit anti-EtBG serum diluted 1:125 in COVAp and incubated for overnight at 4°C. The beads are washed once with 200 µl COVAp and then 3 times with 200 µl PBS, 0.1 % Tween 20 as above and are resuspended in 200 µl PBS, 0.1 % Tween 20. 70 µl of the above bead suspensions (~2 x 10^7) are added to 50 µl of 40 ng/µl FITC-labelled goat anti rabbit Fab (Jackson 115-095-006) in PBS, 0.1 % Tween 20 and incubated 1 hour at room temperature. The beads are washed 3 times with 200 µl PBS, 0.1 % Tween 20 as above, then resuspended in 1 ml PBS, 0.1 % Tween 20 and 10,000 events analysed using a FACScan flow cytometer (Becton Dickinson).

Consequently, as seen in Fig. 16, beads to which genes encoding the phosphotriesterase tagged with the Flag peptide were attached (along with an antibody that binds the Flag peptide) could be clearly distinguished from genes to which other genes, encoding enzymes with no phosphotriesterase activity (e.g., N-Flag-M.HaeIII), were attached.

Example 11.

***E. coli* BirA transcribed and translated in vitro catalyses a reaction which gives rise to a change in the fluorescence properties of substrate-labelled microspheres in the aqueous compartments of a water-in oil emulsion.**

The gene encoding a peptide from *Propionibacterium shermanii* which is biotinylated *in vivo* in *E. coli* is amplified using oligonucleotides BCCP5 and BCCP3 from the vector Pinpoint Xa-1 (Promega). The PCR fragment is cloned into the vector pET-23d(FLAG) digested with *Bam*HI and *Hind*III, downstream of a T7 RNA polymerase promoter and the phage T7 gene 10 translational start site, and in frame with an N-terminal FLAG peptide-coding region; this vector is termed pET-23d(FLAG-BCCP). The vector pET-23d(FLAG) is identical to the vector pET-23d (Novagen) except for the region between the unique *Nco*I and *Bam*HI sites, which has been modified to include an N-terminal FLAG peptide-coding region as shown below in Scheme 2. In order to append a hexahistidine tag to the C-terminus of the protein, the two oligonucleotides BCCPHis+

and BCCPHis- were annealed and then ligated into the vector pET-23d(FLAG-BCCP) digested with *Sac*I and *Not*I, yielding the vector pET-(FLAG-BCCP-His). The protein FLAG-BCCP-His (termed FBH) is overexpressed in strain C41(DE3) (Miroux & Walker, 1996), harvested and purified with Ni-NTA agarose (Qiagen) under native conditions, following the manufacturer's protocol. Biotinylated protein is depleted by incubation with an equal volume of avidin-agarose (Sigma), pre-equilibrated with a wash buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 20 mM imidazole) for 1 hour at 4°C. The suspension is then centrifuged at 10,000 g for 2 minutes and the supernatant retained, aliquoted and stored in liquid nitrogen (long-term) or at 4°C.

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                  M  D  Y  K  D  D  D  D  K  M  H  G  N  E  G
------TATACCATGGACTACAAAGATGACGATGATAAAATGCATGGCAACGAAGGT
pET-23d - NcoI site  (appended FLAG coding region)

 T
ACCGGATCC---------------------------AAGCTT
   BamHI site of pET-23d              HindIII site
```

Scheme 2

The gene encoding *E. coli* BirA was amplified by PCR using oligonucleotides BirA5 and BirA3 from a pBluescript 2SK+ vector containing the *E. coli* BirA gene (gift from P. Wang, unpublished). The PCR fragment is cloned into the vector pGEM-4Z(K2) digested with *Kpn*I and *Xho*I downstream of the *lac* promoter, T7 RNA polymerase promoter and the efficient phage T7 gene 10 translational start site. The vector pGEM-4Z(K2) is identical to the vector pGEM-4Z^{NcoI} (see Example 8, Scheme 1), except for the region between the unique *Nco*I and *Kpn*I sites, which has been modified according to Scheme 3 shown below to contain a unique *Xho*I site downstream of the *Nco*I site.

```
                     M  G  G  S  S
------------CCATGGGGGCTCGAGC---------GGTACC---
pGEM-4Z^NcoI---NcoI       XhoI       KpnI site of pGEM-4Z^NcoI
```

Scheme 3

DNA sequencing identifies a clone with the correct nucleotide sequence, termed pGEM-BirA. The pGEM-BirA plasmid described above is amplified by PCR using primers LMB2 and LMB3 as above to create a 1139 base pair PCR fragment (BirA_LMB2-3) which carries the T7 RNA polymerase promoter, the phage T7 gene 10 translational start site and the BirA gene. The PCR fragment is purified directly using Wizard PCR Preps (Promega).

60 μL aliquots (1.2×10^9 beads) of 1.0 μm diameter nonfluorescent goat anti-mouse IgG labelled microspheres (Bangs Laboratories, CP03N) were spun in a microfuge at approximately 2,600 g (6,000 rpm) for 3 minutes. The supernatant was removed and the beads resuspended in 60 μL 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20, 0.5% BSA. The beads were spun again, resuspended in 60 μL M5 anti-FLAG antibody (Sigma F4042) and incubated overnight at 4°C. The beads were spun again (2,600 g) for 3 minutes, the supernatant was removed, and the beads were resuspended in a mixture of 30 μL 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20, 0.5% BSA and 30 μL of FBH protein obtained as above (final protein concentration approx. 4 mg / ml) and incubated for 1 hour at room temperature.

Meanwhile, 60 μL aliquots of a prokaryotic *in vitro* coupled transcription/translation system designed for linear templates (Lesley et al., 1991) was prepared, using a commercial kit (*E. coli* S30 Extract System for Linear Templates; Promega), supplemented with T7 RNA polymerase (2,000 units), 10 nM BirA_LMB2-3 DNA (or no DNA at all). These aliquots were incubated at 25°C for 1 hour to allow translation.

The 60 μL aliquots of beads were spun at 2,600 g (6,000 rpm) in a microfuge for 3 minutes and the supernatant removed. They were resuspended in 60 μL of 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20, 0.5% BSA, respun and the supernatant removed. Finally they were resuspended on ice in a 54 μL aliquot of the prokaryotic *in vitro* coupled transcription/translation reactions described above, supplemented with 3 μL of 2 mM d-biotin and 3μL of 0.2 M ATP.

A 5 µl aliquot was removed from each reaction mixture and left non-emulsified. 50 µl of the remaining reaction mixture was emulsified essentially as Tawfik & Griffiths (1998).

The oil phase was freshly prepared by dissolving 4.5% (v/v) Span 80 (Fluka) in mineral oil (Sigma, #M-5904) followed by 0.5% (v/v) Tween 80 (SigmaUltra; #P-8074). Ice-cooled reaction mixtures were added gradually (in 5 aliquots of 10 µl over ~2 minutes) to 1.0 ml of ice-cooled oil-phase in a 5 ml Biofreeze Vial (Costar, #2051) whilst stirring with a magnetic bar (8x3 mm with a pivot ring; Scientific Industries International, Loughborough, UK). Stirring (at 1150 rpm) was continued for an additional 1 minute on ice.

All reactions were incubated for 4 hours at 37°C to allow the biotinylation reaction to proceed.

The emulsions were transferred to 1.5 ml microfuge tubes, spun 1 min. 13.5k rpm in a microfuge and the oil phase removed leaving the concentrated (but still intact) emulsion at the bottom of the tube. 200 µl 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20, 0.5% BSA were added and the emulsion broken by extracting 4 times with 1 ml hexane, vortexing between each hexane addition. Residual hexane was removed by spinning for 10 min at ambient temperature under vacuum in a Speedvac (Farmingdale, NY).

Approximately 1 x 10^8 beads from the broken emulsions and the non-emulsified reactions were then washed twice with 100 µl TNT / BSA in a 0.45 µm MultiScreen-HV filter plate (Millipore, MAHVN4510), thoroughly resuspending between each wash. Beads were then resuspend in 50 µl 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20, 0.5% BSA containing 1 µL of a streptavidin-HRP solution (provided with the NEN TSA™-Direct kit) and incubated for 30 minutes at ambient temperature. The beads were washed twice with 100 µl 0.2 M Tris, 10 mM imidazole, pH 8.8, as above, then resuspended in 50 µL 0.2 M Tris, 10 mM imidazole, pH 8.8, 0.01% H_2O_2. 1 µL of a fluorescein tyramide stock solution (made up according to the manufacturer's instructions (NEN TSA™-Direct kit)) was added, and the reaction left to proceed for ten minutes. The beads were washed twice

with PBS, as above, and finally resuspended in a total of 500 μL PBS, transferred to a 5 ml polystyrene round-bottomed tube (Falcon) and 10,000 events analysed using a FACScan flow cytometer (Becton Dickinson).

As can be seen from Fig. 17, both in emulsified and non-emulsified reactions, the reaction catalysed by *in vitro* translated BirA results in beads with higher fluorescence than when no enzyme was present. It appears that beads which have been incubated in an emulsion with *in vitro* translated BirA are more fluorescent than beads which have not been incubated in emulsions.

Example 12

A change in fluorescence of genetic elements can be used to selectively enrich genetic elements encoding peptides with a binding activity. The fluorescently labelled genetic elements are isolated by flow cytometric sorting.

One format for the selection of genetic elements is where the genetic element comprises a gene linked to a microbead, which is translated in a microcapsule, and the translated gene-product is coupled back onto the microbead within the microcapsule. Thus, compartmentalisation leads to the formation of complexes of gene-products attached to the gene encoding them. These complexes can subsequently be selected for binding to a ligand by flow cytometric sorting if the binding interaction results in a change in microbead fluorescence.

pET-23d(FLAG) vector encodes N-terminal FLAG-peptide fused to the polylinker region of pET23d (Novagen). pET23d was digested with Nco I/ BamH I, gel purified and redissolved in water. Two synthetic phosphorylated oligonucleotiodes (Vh Bio Ltd, Newcastle upon Tyne, U.K.), FLAG and FLAGas, were mixed at 1 μM concentration each in water, heated for 3 min at 94°C and allowed to cool to room temperature before being added to the digested vector in the ligation mix. The ligation reaction was used unpurified to transform *E.coli* TG-1. Clones containing the insert were identified by Kpn I

digest and verified by sequencing (Oswel Research Product Ltd, Southampton, U.K.). The polylinker region of pET-23d(FLAG) is as follows:

```
NcoI                                         KpnI
          10        20        30        40        50
CCATGGACTACAAAGATGACGATGATAAAATGCATGGCAACGAAGGTACC
GGTACCTGATGTTTCTACTGCTACTATTTTACGTACCGTTGCTTCCATGG
   M  D  Y  K  D  D  D  D  K
   <     FLAG-peptide tag     >

BamHI EcoRI SacI   SalI  HindIII Not I  XhoI
          60        70        80        90
GGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCA
CCTAGGCTTAAGCTCGAGGCAGCTGTTCGAACGCCGGCGTGAGCTCGT
```

Biotinylated FLAG-HA expression construct was prepared from the pET-23d(FLAG) vector by PCR. The peptide sequence YPYDVPDYA from the influenza haemagglutinin was appended to the FLAG-tag in pET-23d(FLAG) using the primer FLAGHA and the 5'-biotinylated primer pETrev.b. The amplification product is 903 bases long and the coding region of the construct is:

```
         10        20        30        40        50
ATGGACTACAAAGATGACGATGATAAAATGCATGGCAACGAAGGTACCGG
TACCTGATGTTTCTACTGCTACTATTTTACGTACCGTTGCTTCCATGGCC
 M  D  Y  K  D  D  D  D  K  M  H  G  N  E  G  T  G
 <     FLAG-peptide tag     >

         60        70        80        90       100

ATCCGGAGGAGGATATCCGTATGATGTGCCGGATTATGCGGGAGGAGGATCCTAA

TAGGCCTCCTCCTATAGGCATACTACACGGCCTAATACGCCCTCCTCCTAGGATT
```

S G G G Y P Y D V P D Y A G G G S

*

< HA-peptide tag >

The competitor construct in the selection process is *E. coli folA* gene encoding dihydrofolate reductase amplified from pET23a/folA using primers pETfor and pETrev.b.

PCR fragments were gel-purified using QIAquick Gel Extraction kit (Qiagen). DNA concentration was measured by UV spectrophotometry. Dilutions of PCR –prepared expression constructs were made in 0.5 mg/ml carrier DNA prepared from Hind III digested lambda phage DNA (40 min at 80°C, followed by ethanol-precipitation and dissolution in water).

2×10^9 streptavidin-coated 0.95 μm polystyrene beads in a 100 μl aliquot of 1% suspension (Bangs Laboratories, Inc. CP01N) were spun in a microfuge at approximately 2,600 g (6,000 rpm) for 3 minutes. The supernatant was removed and the beads resuspended in 100 μL 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20, 0.5% BSA (TNTB). 7 μl of 2 mg/ml biotinylated anti-FLAG monoclonal antibody M5 (Sigma) was added to the resuspended beads and the mix was incubated at room temperature for two hours. Following coating with the antibody, the beads were washed for three times with 200 μl TNTB, resuspended in 100 μl TNTB and split into 10 μl aliquots 1 and 2 and 40 μl aliquots 3 and 4. 0.7 nM stock solution of either, (#1) pure FLAG-HA DNA,(#2) pure folA DNA, or (#3 and #4) pure FLAG-HA DNA diluted in a 1000 fold excess of folA DNA were prepared in Hind III-digested lambda DNA and applied to the bead aliquots. The binding reaction was allowed to proceed overnight at 4°C. The maximum number of genes per bead was 2 in aliquots 1-3 and 0.2 in aliquot 4. The beads coated with FLAG-HA construct served as positive control and the beads coated with folA as negative control.

#	DNA	Ratio folA: FLAG-HA	Beads	DNA (nM)	DNA (μl)	Molecules of DNA/bead	S30 (μl)	Emulsion (ml)

1	FLAG-HA	-	$2x10^8$	0.7	1	2	25	0.5
2	folA	-	$2x10^8$	0.7	1	2	25	0.5
3	folA:HA	1000:1	$8x10^8$	0.7	4	2	50	2x 0.5
4	folA:HA	1000:1	$8x10^8$	0.7	0.4	0.2	50	2x 0.5

After overnight incubation at 4°C, the beads were washed twice in TNTB and resuspended in S30 *in vitro* translation mixture (S30 Extract System for Linear Templates, Promega) supplemented with T7 RNA polymerase (20 units/ µl).

The ice-cooled *in vitro* translation reactions were added gradually (in 5 aliquots of 10 µl over ~2 minutes) to 0.5 ml of ice-cooled oil-phase (freshly prepared by dissolving 4.5% (v/v) Span 80 (Fluka) in mineral oil (Sigma, #M-5904) followed by 0.5% (v/v) Tween 80 (SigmaUltra; #P-8074)in a 5 ml Costar Biofreeze Vial (#2051)) whilst stirring with a magnetic bar (8x3 mm with a pivot ring; Scientific Industries International, Loughborough, UK). Stirring (at 1150 rpm) was continued for an additional 3 minutes on ice. Reactions were then incubated 90 min at 30°C.

The emulsions were transferred to 1.5 ml microfuge tubes, spun 8 min. 6.5k rpm in a microfuge and the oil phase removed leaving the concentrated (but still intact) emulsion at the bottom of the tube. 200 µl 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20 (TNT) were added and the emulsion broken by extracting 4 times with 1 ml hexane, vortexing between each hexane addition. Residual hexane was removed by bubbling air through the suspension of beads for 1-2 min at ambient temperature.

Beads from the broken emulsions were then washed twice with 100 µl TNT in a 0.45 µm MultiScreen-HV filter plate (Millipore, MAHVN4510), thoroughly resuspending between each wash. Beads were then resuspend in TNTB at 10^6 beads/µl and containing 100 mU/ml rat anti-HA –Peroxidase, High Affinity (3F10) conjugate (Boehringer Mannheim).

The beads were incubated with the antibody for 30 minutes at ambient temperature and washed three times with 200 µl TNT before being resuspended in 2 ml of 0.2 M Tris, 10 mM imidazole, pH 8.8. The suspended beads were sonicated for 1 min on ice using Heat Systems sonicator at power 1, 95% cycle, 3.4 mm tip. The sonicated beads were

resuspended at10^8 beads/ml in 0.2 M Tris, 10 mM imidazole, pH 8.8. To this suspension of beads an equal volume of tyramine signal amplification (TSA) buffer 0.2 M Tris, 10 mM imidazole, pH 8.8, 0.004% H_2O_2, 5 µg/ml fluorescein tyramine was added.

Fluorescein tyramine was synthesised as described by Hopman et al. (Anthon H.N. Hopman, Frans C.S. Ramaekers, Ernst J.M. Speel, The Journal of Histochemistry and Cytochemistry vol 46(6), 771-777, 1998).

The reaction is left to proceed for five minutes at room temperature and stopped by addition of 1/10th of volume of 10% bovine serum albumin in PBS (BSA, Sigma). The beads were spun down in 2 ml aliquots of the labelling reaction and washed 2 times in TNTB and once in PBS. Finally the beads were resuspended in 2 ml of PBS and sonicated as above.

The beads coated with genes encoding folA, FLAG-HA or 1000-fold dilution of FLAG-HA in folA were analysed on a Becton Dickinson FACScan flow cytometer.

In Figure 18, low resolution histogram A demonstrates that the beads carrying FLAG-HA DNA (sample #1) are significantly more fluorescently labelled than the negative control folA (sample #2). The spiked mixtures #3 and #4 run predominantly identically to negative control sample except for a small number of highly fluorescent beads (panel B). 0.04% of beads in sample #3 and 0.02% of beads in sample #4 fell into the region M1 that covers 95% of positive events.

The beads in samples #3 and #4 that fell into region M1 were sorted using a MoFlo fluorescence-activated cell sorter. Two sets of sorted beads were acquired for both samples #3 and #4. In set one 500 beads were collected into a single tube. In set two 96 beads were collected individually into the wells of a 96-well plate. Both sets of beads were subjected to 35-cycle PCR using primers pETrev.b and FLAGrev1.

The amplification products were analysed by gel electrophoresis (Figure 19). The product sizes are 903 bases for FLAG-HA and 1390 bp for folA.

The gel electrophoretic analysis of the amplification reaction products suggests significant enrichment during the course of sorting. In panel A there are no FLAG-HA bands visible on the lanes of the products amplified from unsorted reactions #3 and #4 whereas the FLAG –HA band in the samples from the sorted beads is strongly visible. Definitive data regarding the nature of the amplified DNA were obtained from the analysis of DNA amplified from single beads. In total 22 beads out of 96 yielded a DNA product for reaction #3 and 50% of these were pure FLAG-HA. For reaction #4 9 beads yielded products and 8 were FLAG-HA.

Single-bead data for reaction #3 suggests that at the concentration applied, nominally 2 DNA molecules/bead, most of the beads in fact have only one gene attached allowing unambiguous linkage between the gene and its product. Relatively high number of positively labelled beads meant however that about 50% of the beads recovered were false positives. In sample #4 where there were only ≈0.1 genes/bead the purity of the recovered DNA approached 90%, indicating nearly 1000-fold enrichment in one step.

Oligonucleotides

EDHFR-Fo	5'-CGA GCT AGA GGT ACC TTA TTA CCG CCG CTC CAG AAT CTC AAA GCA ATA G-3'
EDHFR-Ba	5'-GCA TCT GAC AAG CTT AAT AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG ATC AGT CTG ATT GCG GCG TTA GCG GTA G-3'
LMB2-Biotin	5'-Biotin-GTA AAA CGA CGG CCA GT-3'
folA-FW	5'-GCG CGA AGC TTC GAT CAG TCT GAT TGC GGC G-3'
folA-BW	5'-GCG CCT CGA GTT CCG CCG CTC CAG AAT CTC-3'
pETfor.b	5'-Biotin-GAC TCC AAC GTC AAA GGG CG-3'
pETrev.b	5'-Biotin-GGT TTT CAC CGT CAT CAC CG-3'
GFP-FW	5'-GCG CGA AGC T TCG AGT AAA GGA GAA GAA CTT TTC-3'
GFP-BW	5'-GCG CCT CGA GTT TTG TAT AGT TCA TCC ATG CCA TG-3'
GSTM2-2Fo	5'-TGA TGC CGG TAC CTT ATT ACT TGT TGC CCC AGA CAG CC-3'
GSTM2-2Ba	5'-AGT TAA GTC TAA GCT TAA TAA TTT TGT TTA ACT TTA AGA AGG AGA TAT ACA TAT GCC CAT GAC ACT GGG GTA C-3'
LMB2	5'-GTA AAA CGA CGG CCA GT-3'
LMB3	5'-CAG GAA ACA GCT ATG AC-3'
N-Flag-OPD-Fo	5'-TCG ATA CGT CGG TAC CTT ATT ATG ACG CCC GCA AGG TCG GTG-3'
N-Flag-OPD-Bc	5'-CAT TGC CAA GCC ATG GAC TAC AAA GAT GAC GAT GAT AAA ATC ACC AAC AGC GGC GAT CGG ATC AAT ACC G-3'

BCCP5	5'-CTA GGT CAT GGA TCC ATG AAA CTG AAG GTA ACA GTC AAC GGC-3'
BCCP3	5'-CAG ATA GCT AAG CTT TTA TTA TTC GAT GAG CTC GAG ATC CCC-3'
BCCPHis+	5'-CAT CGA AGG TGG CAG CTC TGC-3'
BCCPHis-	5'-GGC CGC AGA GCT GCC ACC TTC GAT GAG CT-3'
BirA5	5'-ATC GTA GCA CTC GAG CAT GAA GGA TAA CAC CGT GCC A-3'
BirA3	5'-GTC ATG ACT GGT ACC TTA TTA TTT TTC TGC ACT ACG CAG-3'
FLAG	5'-CAT GGA CTA CAA AGA TGA CGA TGA TAA AAT GCA TGG CAA CGA AGG TAC CG-3'
FLAGas	5'-GAT CCG GTA CCT TCG TTG CAT GCA TTT TAT CAT CGT CAT CTT TGT AGT C-3'
FLAGHA	5'-AAC TCA GCT TCC TTT CGG GCT TTG TTA GGA TCC TCC TCC CGC ATA ATC CGG CAC ATC ATA CGG ATA TCC TCC TCC GGA TCC GGT ACC TTC GTT GCC-3'
pETrev.b	5'-biotin-GGT TTT CAC CGT CAT CAC CG-3'
pETfor	5'-GAC TCC AAC GTC AAA GGG CG-3'
FLAGrev1	5'-AAC TCA GCT TCC TTT CGG GC-3'

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CLAIMS

1. A method for isolating one or more genetic elements encoding a gene product having a desired activity the expression of which may result, directly or indirectly, in the modification of an optical property of a genetic element encoding the gene product, comprising the steps of:

(a) compartmentalising genetic elements into microcapsules;

(b) expressing the genetic elements to produce their respective gene products within the microcapsules;

(c) sorting the genetic elements which produce the gene product(s) having the desired activity according to a change in the optical properties of the genetic elements.

2. A method according to claim 1, wherein in step (b) the activity of the desired gene product within the microcapsule results, directly or indirectly, in the modification of the genetic element encoding the gene product to enable the isolation of the genetic element.

3. A method according to claim 2, wherein in the modification of the genetic element within the microcapsule induces a change in its optical properties.

4. A method according to claim 2, wherein the modification of the genetic element enables it to be further modified outside the microcapsule so as to induce a change in its optical properties.

5. A method according to claim 2, wherein a part of the genetic element is a ligand and the desired gene product within the microcapsule binds, directly or indirectly, to said ligand to enable the isolation of the genetic element.

6. A method according to claim 5, wherein the ligand is also encoded by the genetic element.

7. A method according to claim 2, wherein a part of the genetic element is a substrate and the activity of the desired gene product within the microcapsule results, directly or

indirectly, in the conversion of said substrate into a product which remains part of the genetic element and enables its isolation.

8. A method according to claim 2, wherein the product of the activity of the desired gene product within the microcapsule results, directly or indirectly, in the generation of a product which is subsequently complexed with the genetic element and enables its isolation.

9. A method according to any preceding claim wherein the activity of the desired gene product within the microcapsule results, directly or indirectly, in the alteration of the expression of a second gene within the compartment and the activity of the product of the said second gene enables the isolation of the genetic element using a change in the optical properties of the genetic element.

10. A method according to claim 1, wherein step (b) comprises:
expressing the genetic elements to produce their respective gene products within the microcapsules, linking the gene products to the genetic elements encoding them and isolating the complexes thereby formed.

11. A method according to claim 10, wherein in step (c) the complexes are directly sorted based on their changed optical properties to isolate genetic elements encoding a gene product having the desired activity.

12. A method according to claim 10, wherein in step (c) the complexes are further reacted to induce a conditional change in optical properties of the genetic element dependent on the presence of gene products with the desired activity in the complex.

13. A method according to claim 10, wherein the complexes are subjected to a further compartmentalisation step in order to isolate the genetic elements encoding a gene product having the desired activity.

14. A method according to claim 1, wherein the change in optical properties of the genetic element is due to binding of a gene product with distinctive optical properties to the genetic element.

15. A method according to claim 1, wherein the change in optical properties of the genetic element is due to binding of a ligand with distinctive optical properties by the gene product.

16. A method according to claim 1, wherein the change in optical properties of the genetic element is due to a change in the optical properties of the gene product when bound to ligand.

17. A method according to claim 1, wherein the change in optical properties of the genetic element is due to a change in the optical properties of the ligand when bound by the gene product.

18. A method according to claim 1, wherein the change in optical properties of the genetic element is due to a change in the optical properties of both ligand and gene product on binding.

19. A method according to claim 1, wherein the change in optical properties of the genetic element is due to the different optical properties of the substrate and the product of the reaction being selected.

20. A method according to claim 1, wherein both substrate and product have similar optical properties, but only the product, and not the substrate of the reaction being selected binds to, or reacts with, the genetic element, thereby changing the optical properties of the genetic element.

21. A method according to claim 1, wherein further reagents specifically bind to, or specifically react with, the product (and not the substrate) attached to the genetic element, thereby altering the optical properties of the genetic element.

22. A method according to any preceding claim, wherein a non-desired activity of a gene product results in a change in the optical properties of the genetic element which is distinct from that resulting from the desired activity.

23. A method according to claim 22, wherein the optical change resulting from the non-desired activity is used to negatively select the genetic elements.

24. A method according to claim 22, wherein negative selection is combined with positive selection to improve reaction specificity.

25. A method according to claim 24, wherein the improved reaction specificity is an improvement in binding specificity.

26. A method according to claim 24, wherein the improved reaction specificity is an improvement in regio- and/or stereo-selectivity for substrate and/or product.

27. A method according to any preceding claim, wherein the genetic elements are isolated from a library of genetic elements encoding a repertoire of gene products.

28. A method according to any preceding claim, wherein each genetic element encodes more two or more genes and each gene product must have a desired activity in order for the optical properties of the genetic element to be modified to enable them to be sorted.

29. A method according to any preceding claim, wherein each genetic element encodes two or more genes and the gene products must bind to each other in the microcapsule in order for the optical properties of the genetic element to be modified and the genetic elements sorted.

30. A method according to any preceding claim further comprising the additional step of:

(d) introducing one or more mutations into the genetic element(s) isolated in step (c).

31. A method according to any preceding claim further comprising iteratively repeating one or more of steps (a) to (d).

32. A method according to any preceding claim further comprising amplifying the genetic elements.

33. A method according to claim 1, wherein microencapsulation is achieved by forming a water-in-oil emulsion of the aqueous solution in an oil-based medium.

34. A method according to claim 1, wherein the genetic element comprises the gene attached to a microbead.

35. A method according to claim 1, wherein the microbead is nonmagnetic, magnetic or paramagnetic.

36. A method according to claim 1, wherein the genetic elements are sorted by detection of a change in their fluorescence.

37. A method according to claim 36, wherein the sorting of genetic elements is performed using a fluorescence activated cell sorter (FACS) (or similar device).

38. A method according to claim 36, wherein the different fluorescence properties of the substrate and the product are due to fluorescence resonance energy transfer (FRET).

39. A method according to any preceding claim, wherein the internal environment of the microcapsules is modified by the addition of one or more reagents to the oil phase.

40. A method according to any preceding claim, wherein genetic elements modified directly or indirectly by the activity of the desired gene product are further modified by Tyramide Signal Amplification (TSA™; NEN), resulting directly or indirectly in a change in the optical properties of said genetic elements thereby enabling their separation.

41. A product when isolated according to the method of any preceding claim.

42. A method for preparing a gene product, comprising the steps of:
(a) preparing a genetic element encoding the gene product;
(b) compartmentalising genetic elements into microcapsules;
(c) expressing the genetic elements to produce their respective gene products within the microcapsules;
(d) sorting the genetic elements which produce the gene product(s) having the desired activity using a change in their optical properties; and
(e) expressing the gene product having the desired activity.

43. A method for screening a compound or compounds capable of modulating the activity of a gene product, comprising the steps of:
(a) preparing a repertoire of genetic elements encoding gene product;
(b) compartmentalising the genetic elements into microcapsules;
(c) expressing the genetic elements to produce their respective gene products within the microcapsules;
(d) sorting the genetic elements which produce the gene product(s) having the desired activity using a change in their optical properties; and
(e) contacting a gene product having the desired activity with the compound or compounds and monitoring the modulation of an activity of the gene product by the compound or compounds.

44. A method for preparing a compound or compounds comprising the steps of:
(a) providing a synthesis protocol wherein at least one step is facilitated by a polypeptide;
(b) preparing genetic elements encoding variants of the polypeptide which facilitates this step;
(c) compartmentalising the genetic elements into microcapsules;
(d) expressing the genetic elements to produce their respective gene products within the microcapsules;

(e) sorting the genetic elements which produce polypeptide gene product(s) having the desired activity using a change in their optical properties; and

(f) preparing the compound or compounds using the polypeptide gene product identified in (e) to facilitate the relevant step of the synthesis.

FIGURE 1

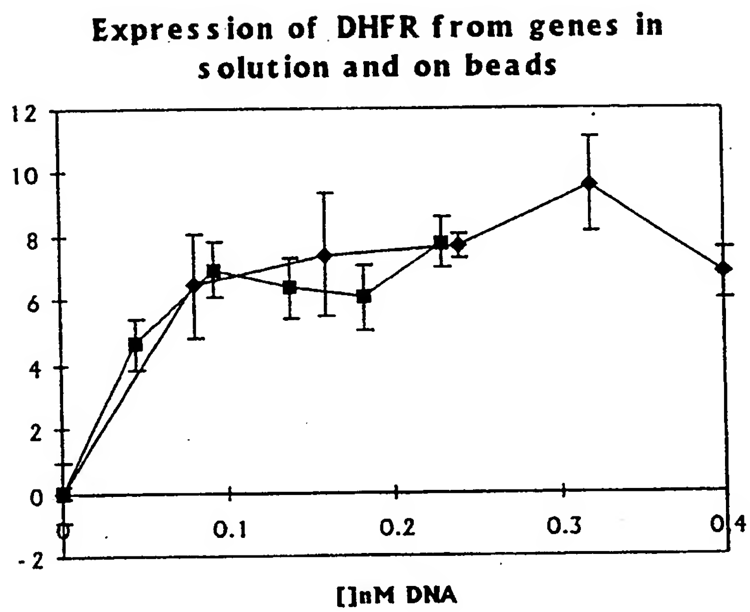
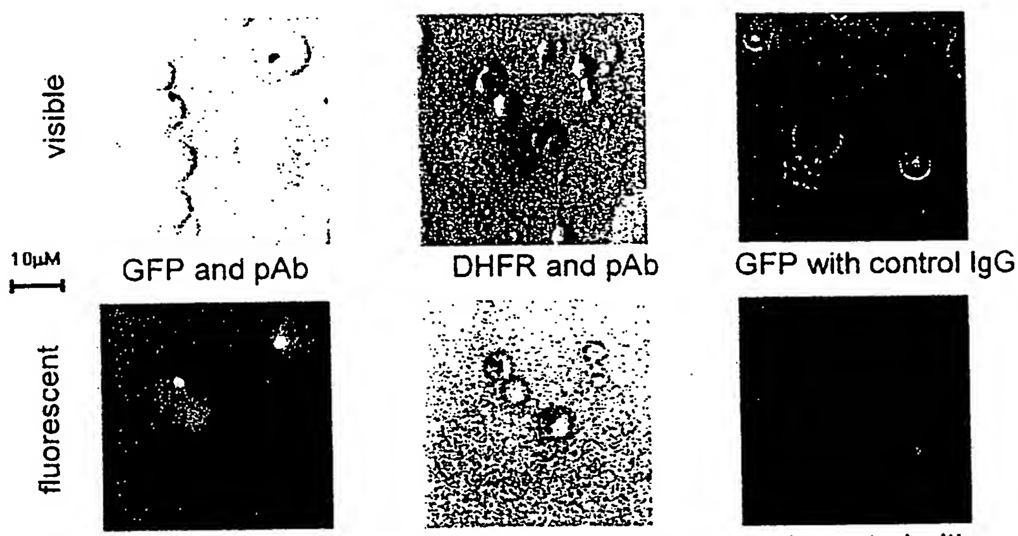

Expression of DHFR from genes in solution and on beads
12
10
8
6
4
2
0
-2
0
0.1
0.2
0.3
0.4
[]nM DNA

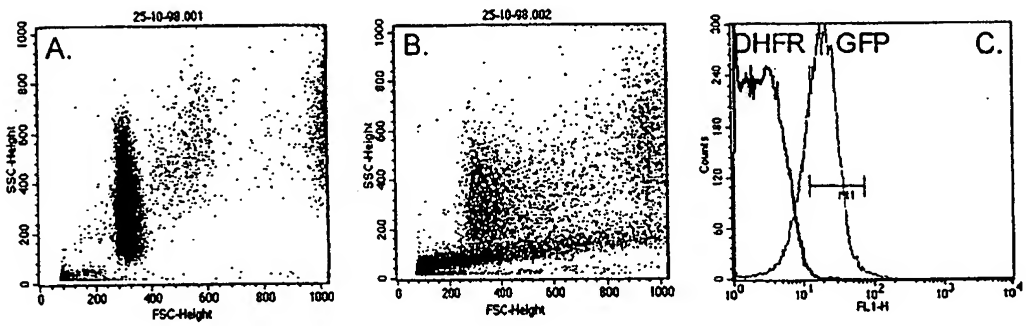
FIGURE 2



Beads coated with biotinylated protein A and rabbit polyclonal a-GFP antibody

Beads coated with biotinylated protein A and rabbit IgG

FIGURE 3

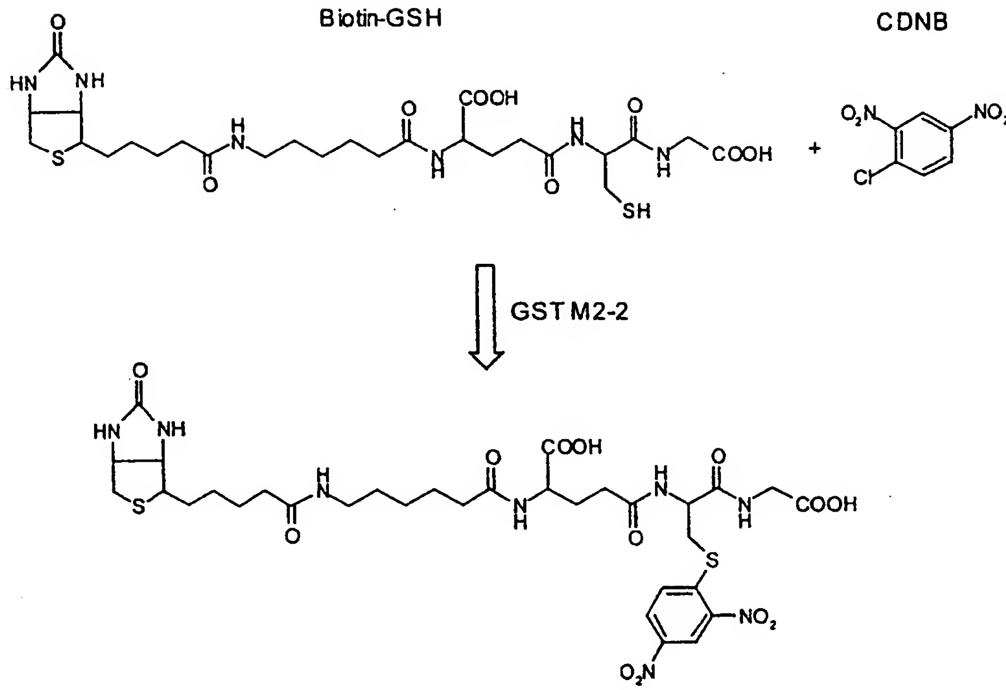


Beads before reaction

Beads after reaction

Fluorescence of beads recovered from emulsion

FIGURE 4



Biotin-GS-DNP

FIGURE 5

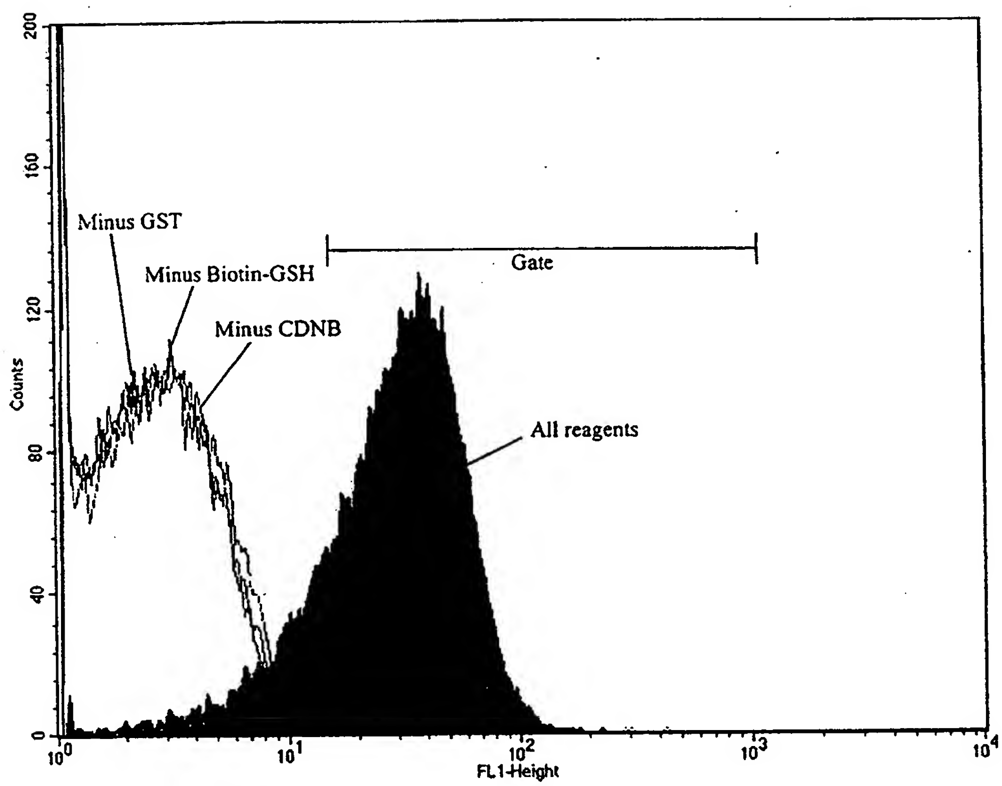
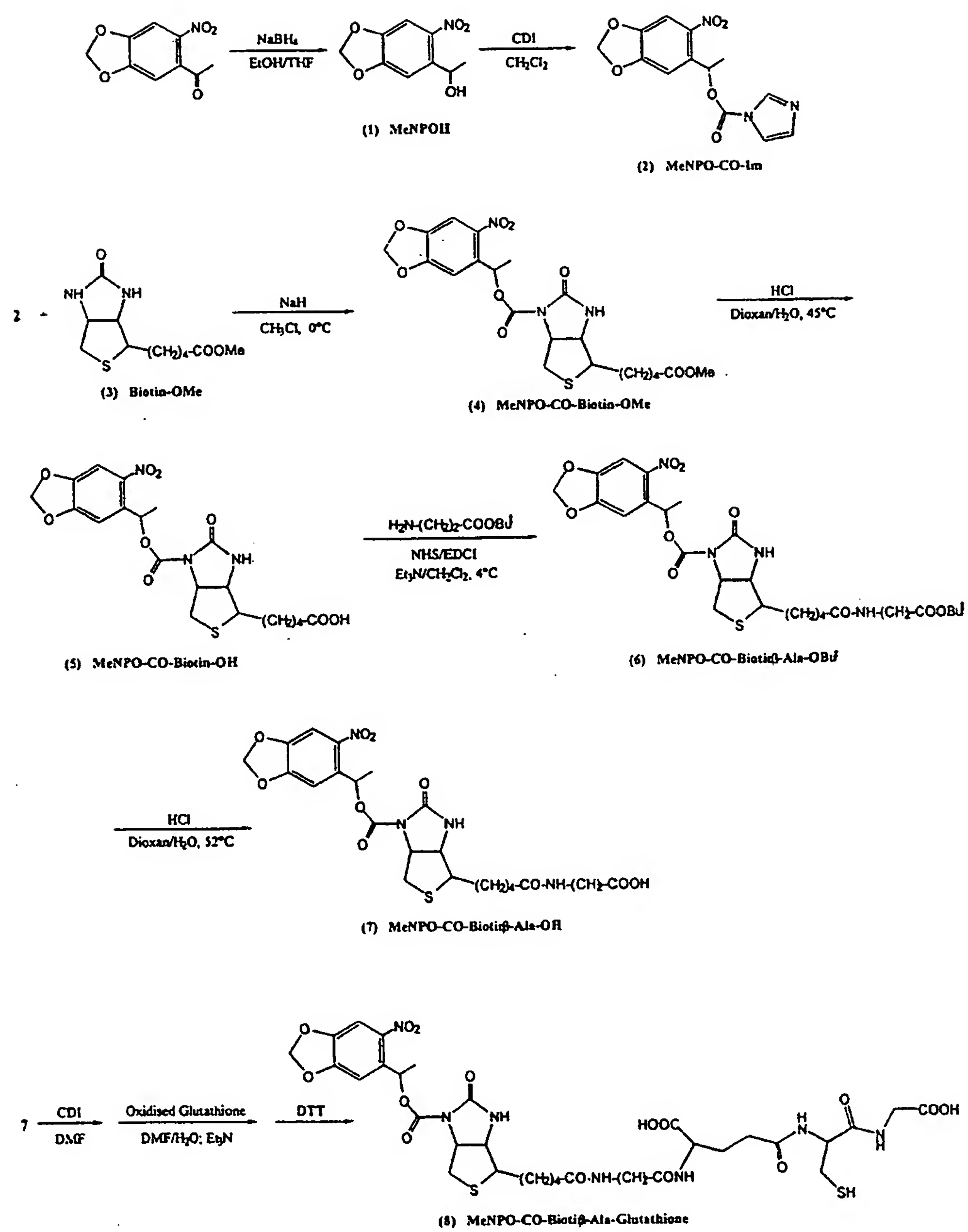
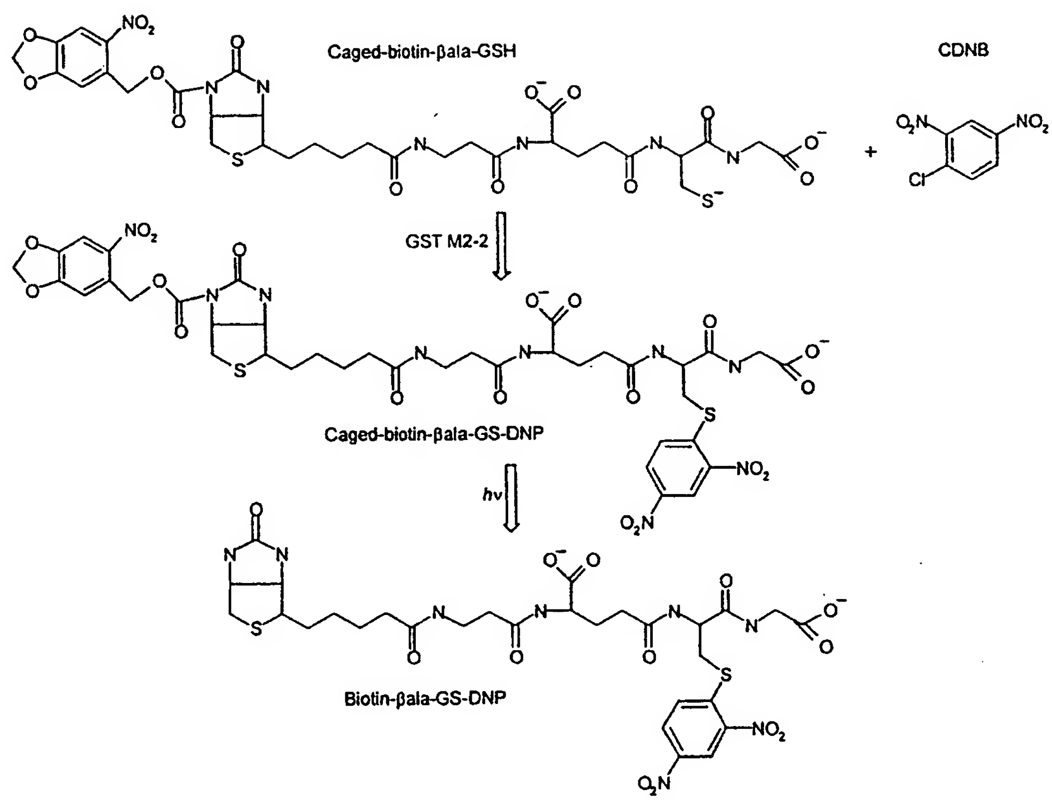

Minus GST
Minus Biotin-GSH
Minus CDNB
Gate
All reagents
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
FL1-Height

FIGURE 6



NaBH4
EtOH/THF
CDI
CH2Cl2
(1) MeNPOH
(2) MeNPO-CO-Im
NaH
CH2Cl, 0°C
HCl
Dioxan/H2O, 45°C
(3) Biotin-OMe
(4) MeNPO-CO-Biotin-OMe
H2N-(CH2)2-COOBu
NHS/EDCI
Et3N/CH2Cl2, 4°C
(5) MeNPO-CO-Biotin-OH
(6) MeNPO-CO-Biotinβ-Ala-OBu
HCl
Dioxan/H2O, 52°C
(7) MeNPO-CO-Biotinβ-Ala-OH
CDI
DMF
Oxidised Glutathione
DMF/H2O: Et3N
DTT
(8) MeNPO-CO-Biotinβ-Ala-Glutathione

FIGURE 7



Caged-biotin-βala-GSH
CDNB
+
GST M2-2
Caged-biotin-βala-GS-DNP
hv
Biotin-βala-GS-DNP

FIGURE 8

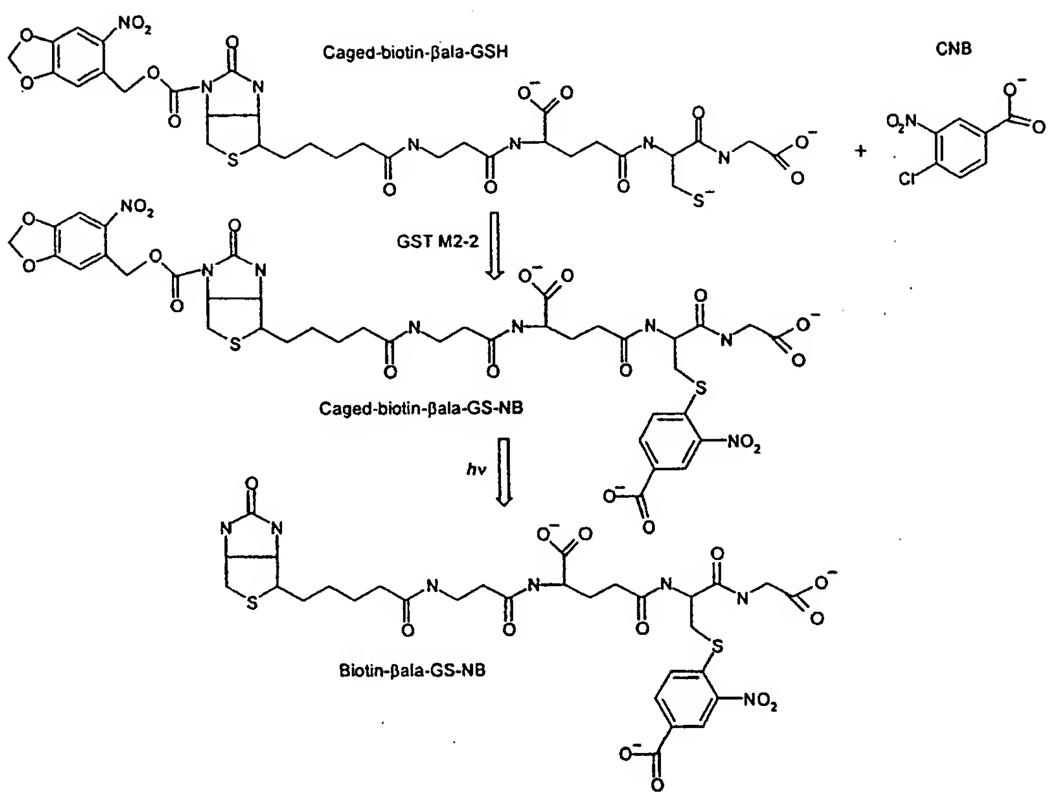

Caged-biotin-βala-GSH
CNB
GST M2-2
Caged-biotin-βala-GS-NB
hv
Biotin-βala-GS-NB

FIGURE 9

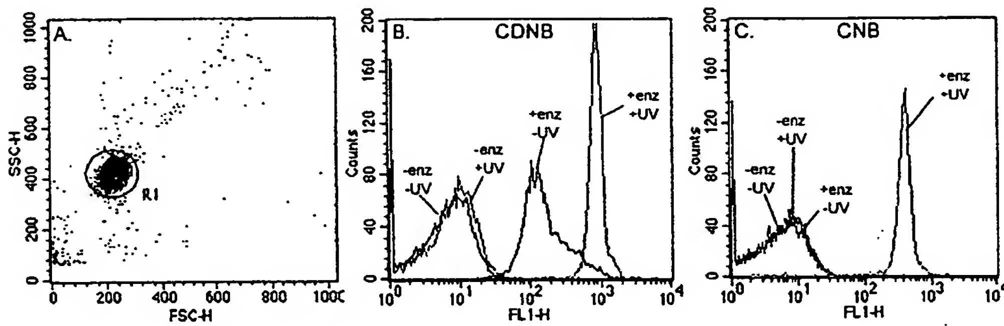

A.
SSC-H
FSC-H
R1
B.
CDNB
Counts
FL1-H
-enz
-UV
-enz
+UV
+enz
-UV
+enz
+UV
C.
CNB
Counts
FL1-H
-enz
-UV
-enz
+UV
+enz
-UV
+enz
+UV

FIGURE 10

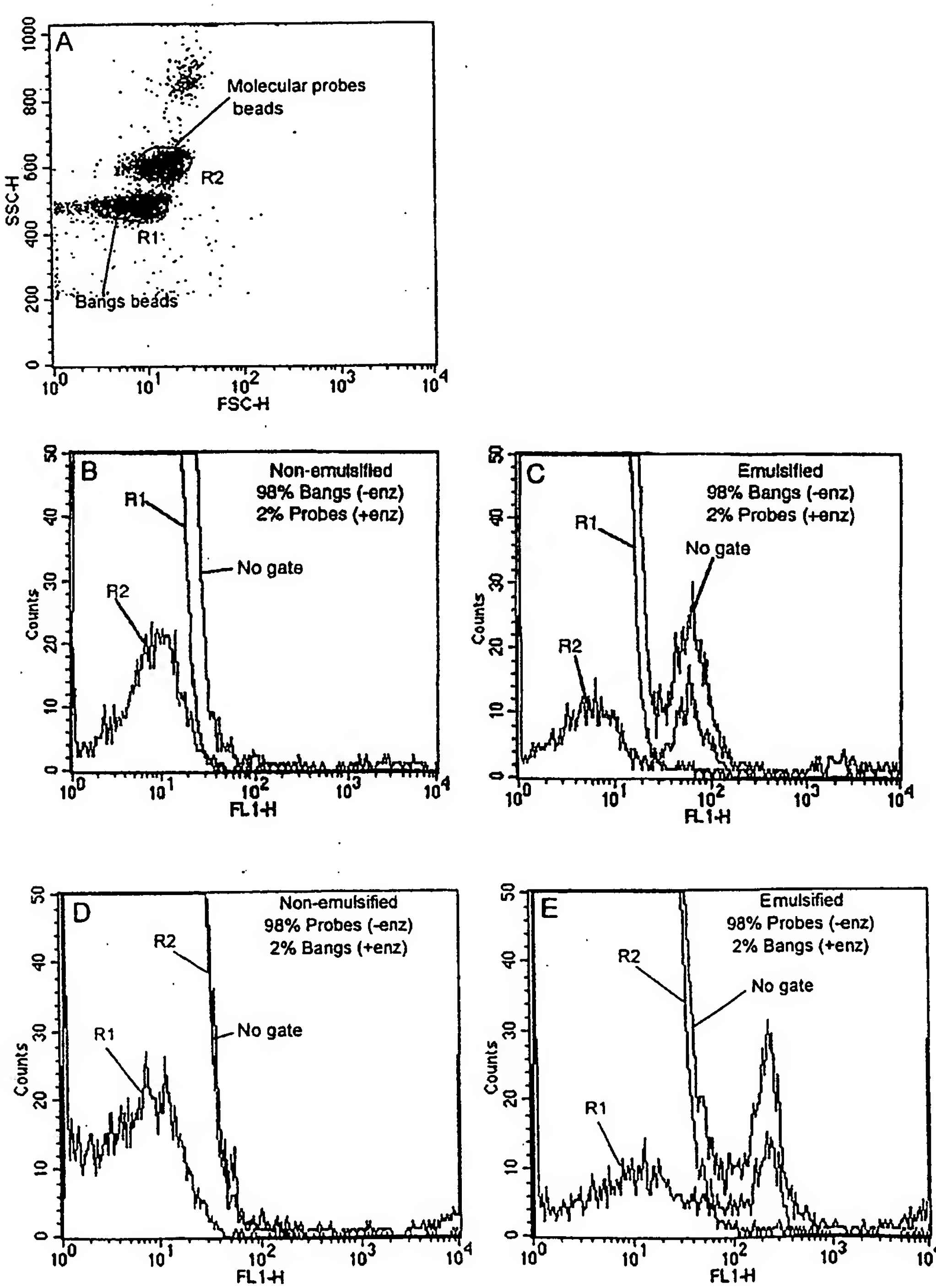

A
Molecular probes beads
R2
R1
Bangs beads
SSC-H
FSC-H
B
Non-emulsified
98% Bangs (-enz)
2% Probes (+enz)
R1
No gate
R2
Counts
FL1-H
C
Emulsified
98% Bangs (-enz)
2% Probes (+enz)
R1
No gate
R2
Counts
FL1-H
D
Non-emulsified
98% Probes (-enz)
2% Bangs (+enz)
R2
No gate
R1
Counts
FL1-H
E
Emulsified
98% Probes (-enz)
2% Bangs (+enz)
R2
No gate
R1
Counts
FL1-H

FIGURE 11

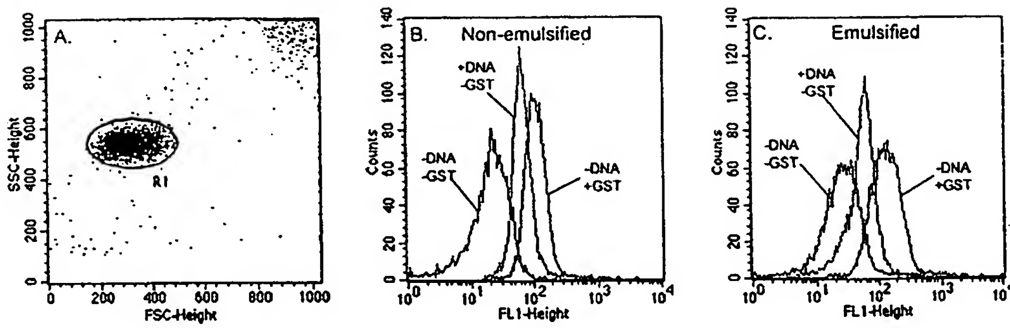

A.
R1
SSC-Height
FSC-Height
B. Non-emulsified
+DNA
-GST
-DNA
-GST
-DNA
+GST
Counts
FL1-Height
C. Emulsified
+DNA
-GST
-DNA
-GST
-DNA
+GST
Counts
FL1-Height

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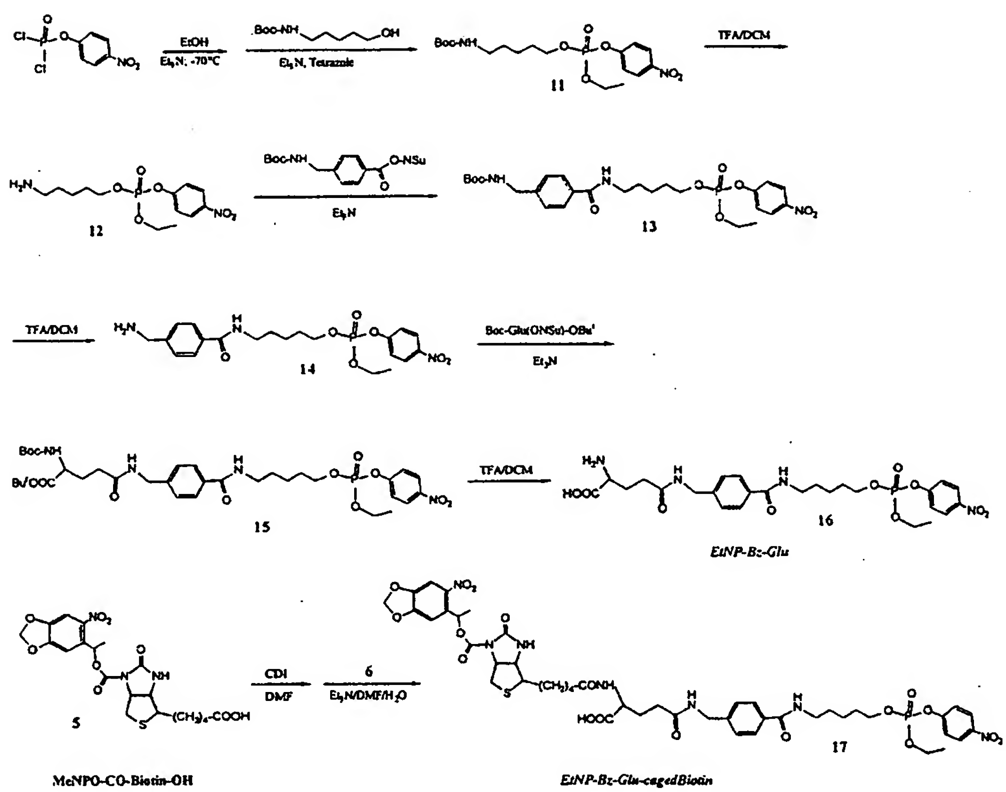
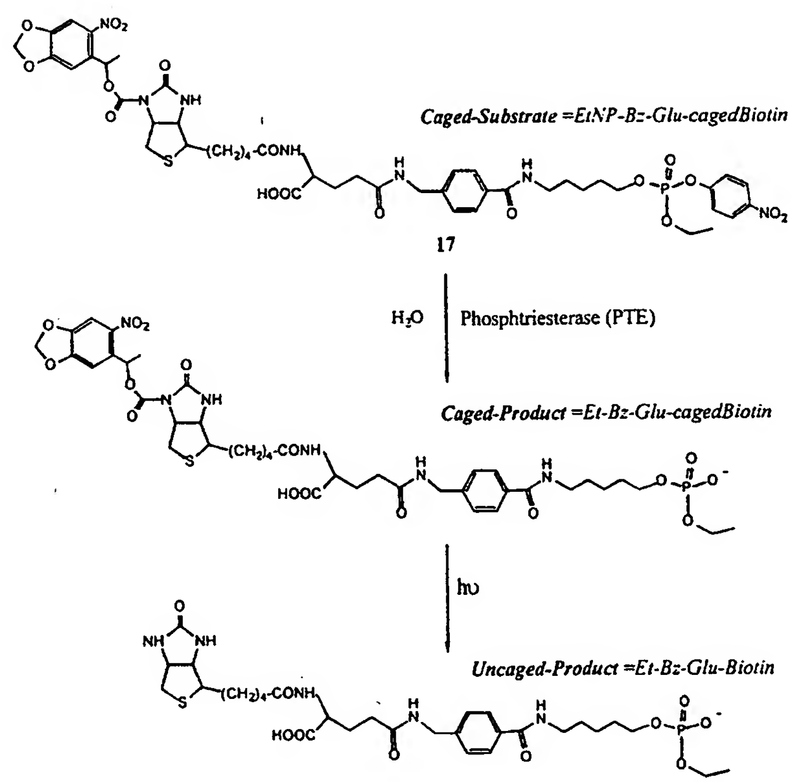
FIGURE 12



13/19

FIGURE 13



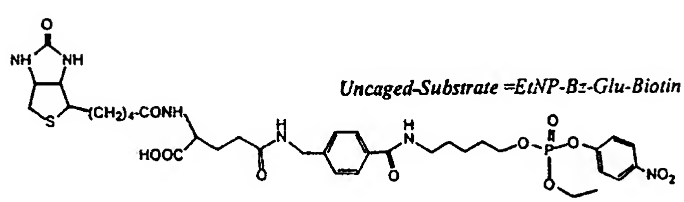
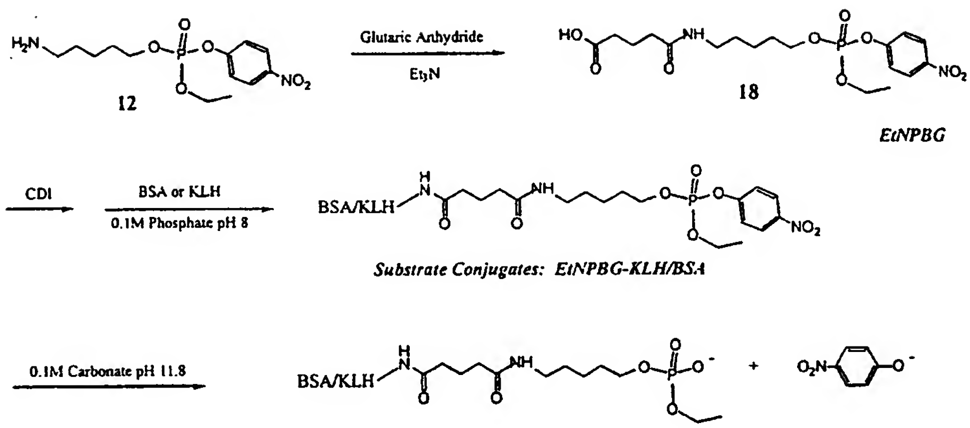


FIGURE 14



Substrate Conjugates: EtNPBG-KLH/BSA

Product Conjugates: EtBG-KLH/BSA

FIGURE 15

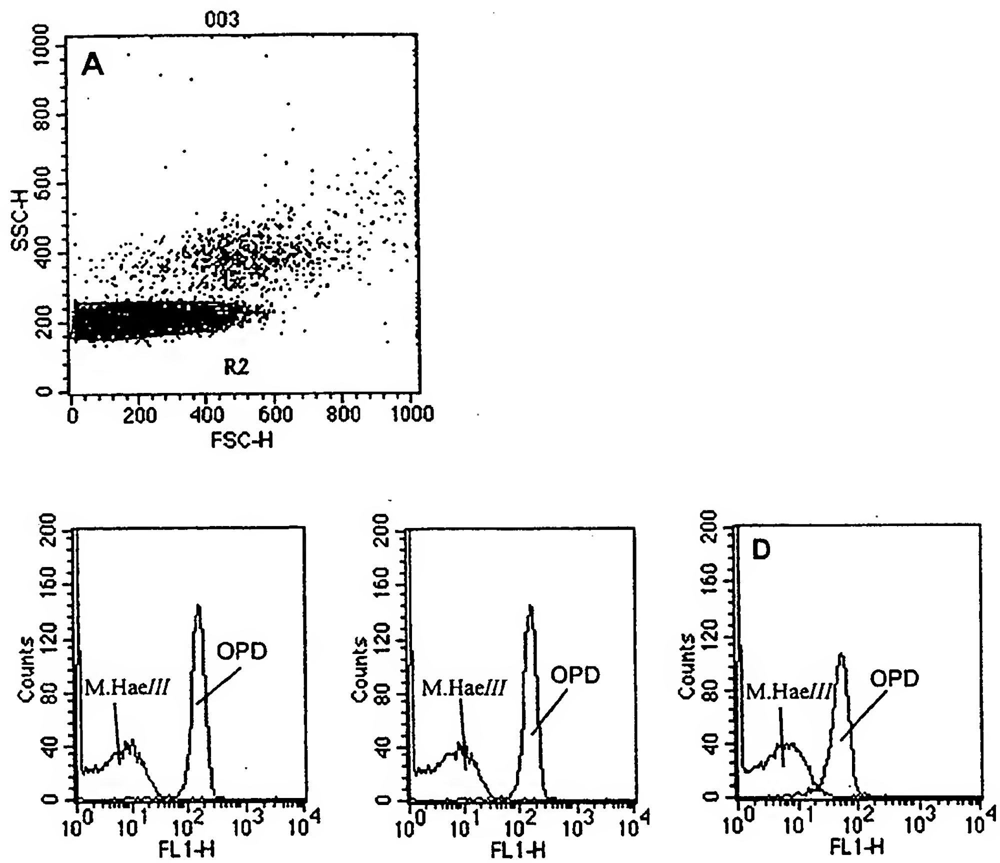
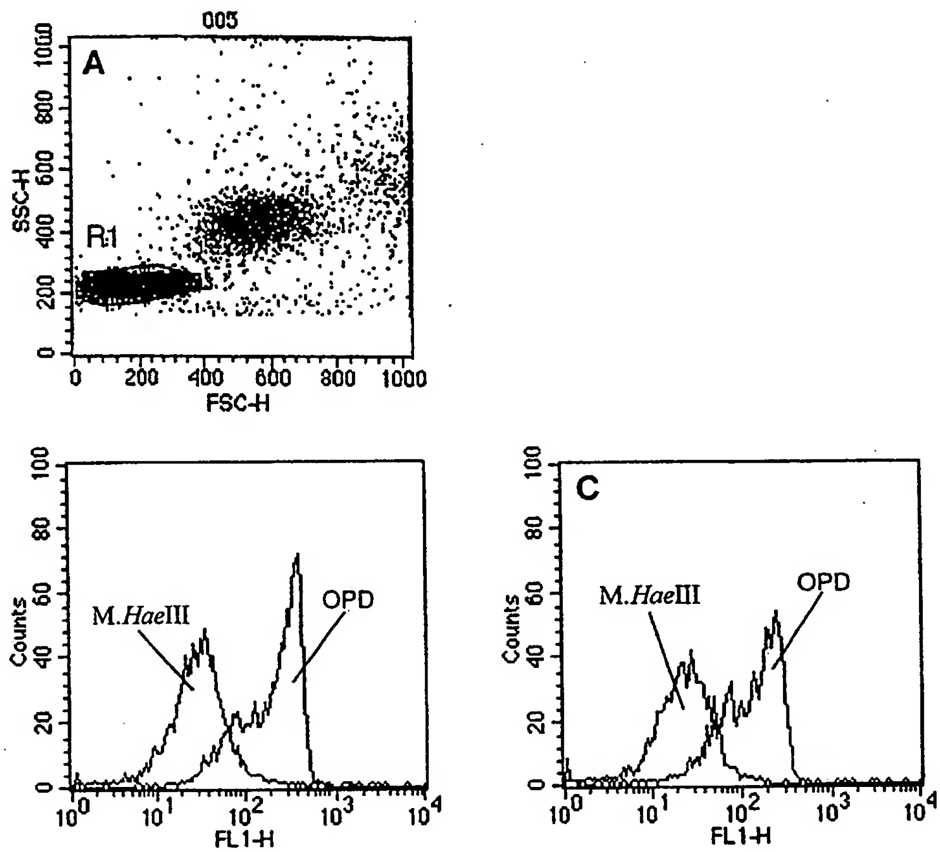


FIGURE 16



005
A
SSC-H
FSC-H
R1
0
200
400
600
800
1000
Counts
FL1-H
M.*Hae*III
OPD
C

FIGURE 17

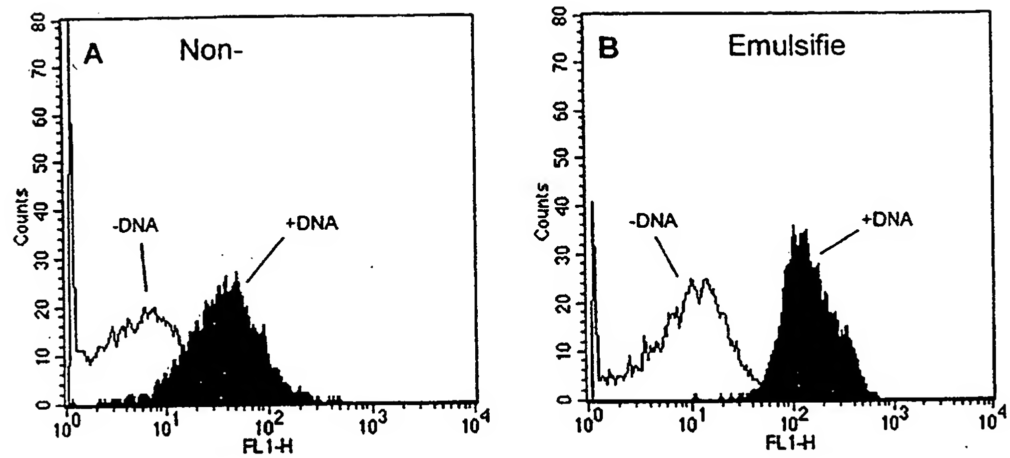


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FIGURE 18

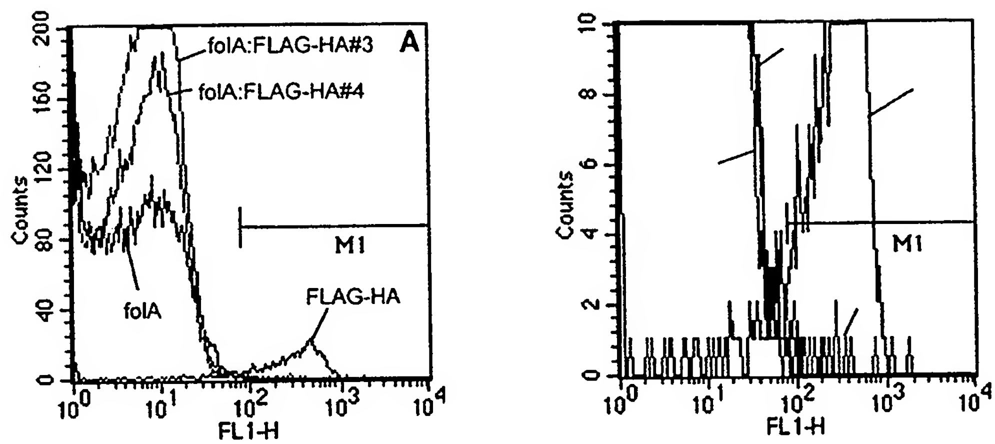
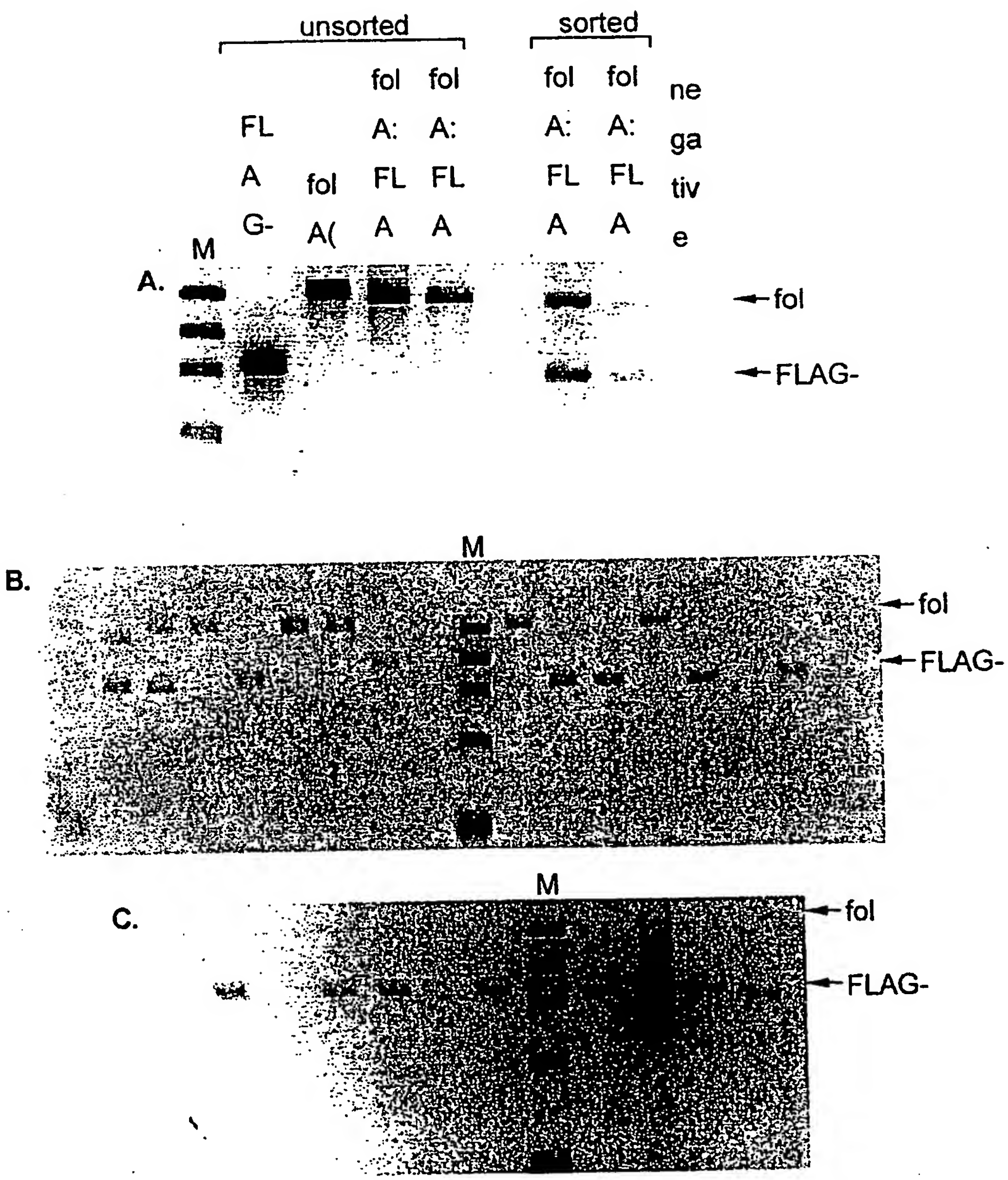


FIGURE 19


unsorted
sorted
FL A G-
fol A(
fol A: FL A
fol A: FL A
fol A: FL A
fol A: FL A
ne ga tiv e
M
A.
fol
FLAG-
B.
M
fol
FLAG-
C.
M
fol
FLAG-

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/00030

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	D S TAWFIK AND A D GRIFFITHS: "Man-made cell-like compartments for molecular evolution" NATURE BIOTECHNOLOGY,US,NATURE PUBLISHING, vol. 7, no. 16, 1 July 1998 (1998-07-01), pages 652-656, XP002079691 ISSN: 1087-0156 cited in the application the whole document	1-3, 5-11,14, 19, 22-27, 32,33, 36,37,41
P,X	WO 99 02671 A (MEDICAL RES COUNCIL ;TAWFIK DAN (GB); GRIFFITHS ANDREW (GB)) 21 January 1999 (1999-01-21) cited in the application claims 1-29; figures 1-3	1-3, 5-11,14, 19, 22-27, 32,33, 36,37,41

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 2000	26/04/2000

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/00030

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9902671 A	21-01-1999	AU 8123198 A	08-02-1999
		GB 2342094 A	05-04-2000

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WO 01/18244 A2

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9921155.9 8 September 1999 (08.09.1999) GB

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(74) Agents: MASCHIO, Antonio et al.; D Young & Co, 21 New Fetter Lane, London EC4A 1DA (GB).

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Published:
— *Without international search report and to be republished upon receipt of that report.*

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WO 01/18244 A2

(54) Title: SELECTION SYSTEM

(57) Abstract: A genetic selection system is described based on hybrid σ54 RNAP activator proteins. The invention permits the detection and screening for protein:DNA and protein:protein binding events.

Selection System

The present invention relates to a screening system useful for screening repertoires of DNA binding domains. In particular the invention relates to a screening system based on transcriptional activators of bacterial σ54-dependent promoters.

The majority of proteins involved in cellular functions do so by interacting with other proteins or nucleic acid sequences within the cell. Several approaches have been described that allow the *in vivo* selection of nucleic acids which express polypeptides capable of binding to proteins or DNA in the cell. Arguably the most powerful approaches are the yeast one- and two hybrid systems (Fields S. & Song O. (1989) *Nature* 340, 245; see US Patent 5,283,173, incorporated herein by reference in its entirety) for the screening of protein-DNA and protein-protein interactions, respectively. However, the two-hybrid system requires an eukaryotic host and consequently the diversity that can be screened is limited. Furthermore the system notoriously suffers from an abundance of false positives.

Larger molecular repertoires can be prepared in bacterial hosts and a number of bacterial systems for the screening of protein-protein and protein-DNA interactions have also been reported. Two systems have been put forward in which the polypeptide chain of an enzyme is expressed in two parts fused to two candidate polypeptides. and in which interaction between the candidate polypeptides reconstitutes the function of the enzyme (Karimova G. et al (1998) *Proc. Nat. Acad. Sci USA* 95, 5752; Pelletier J.N. et al (1998) *Proc. Nat. Acad. Sci USA* 95, 12141).

Several in vivo screens for DNA-binding proteins have also been reported (reviewed in Mossing M.C., Bowie J.U. & Sauer R.T. (1991) *Methods Enzymol.* 208, 604; Elledge S.J. et al (1989) *Proc. Nat. Acad. Sci USA* 86, 3689). Each of these methods involves the blockage of a hybrid σ70 promoter by the DNA binding protein. Repression of the promoter either prevents the production of conditionally toxic gene or alleviates repression of an antibiotic gene by transcriptional interference. The transcriptional interference assay (Elledge *et al.*) has been used successfully in one case to select DNA binding proteins with altered specificity (Sera T. & Schultz P.G. (1996) *Proc. Nat. Acad. Sci USA* 93, 2920).

Another σ70-based system utilises recruitment of the polymerase to the promoter by way of a protein-protein interaction between a protein domain fused to the RNA polymerase α subunit and another fused to the lambda repressor bound immediately upstream of the RNA polymerase promoter binding site (Dove S.L., Joung J.K., Hochschild A. (1997), *Nature* 386, 627). By replacing the lambda repressor DNA binding domain with a library of Zn-finger domains, specific DNA binding Zn-finger domains were selected (Joung J.K., Ramm E.I., Pabo C.O. (2000) *Proc Natl Acad Sci U S A*, 97, 7382)

The alternative holoenzyme form of bacterial RNA polymerase (RNAP) contains the σ 54 factor (σ 54-RNAP). As has been previously shown, this polymerase, in most cases, forms a closed complex with the promoter. Unlike σ70 promoters at which the RNA polymerase is bound in an active form and is largely controlled by repression, the σ54 RNA polymerase holoenzyme is transcriptionally incompetent and is unable to initiate transcription by itself. Initiation of transcription requires the presence of a transcriptional activator that catalyses the isomerisation of the closed promoter complex to an open one. Typically, activator proteins bind to a specific upstream activation sequence (UAS) located 80 to 200 bp upstream of the σ 54 core promoter. The function of the UAS is to tether the activator in the right position and to bring it in the vicinity of the promoter in order to increase the efficiency of interaction between the σ 54 RNAP and the activator. Transcriptional activators of σ54 dependent promoters have been called bacterial enhancers because their mechanism of activation is superficially similar to the activation of transcription by enhancer proteins in eukaryotes (Kustu S. et al (1991) *Trends Biochem Sci* 16, 397).

Conversion of the σ 54 RNAP into an active form is catalysed by the binding of an enhancer protein coupled to hydrolysis of ATP. This unusual mechanism accounts for the low level of background transcription and the enormous difference (10^4-10^5) between on and off states in the strongest σ54 promoters effected by a single factor. In comparison, activators of σ70 promoters such as CAP or λcI increase transcription levels usually by less than 10-fold.

Transcriptional activators of σ54 promoters (also known as enhancer-binding proteins or EBPs) share a common structure (see Morrett and Segovia. (1993) J. Bacteriol. 6067-6074) comprising a non-conserved N-terminal domain which has a putative regulatory function, a central domain which is responsible for transcriptional activation, and a C-terminal DNA binding domain which binds the relevant UAS in the target gene. The domains are modular: the central and N-terminal domains together are capable of constitutive activation of σ54 RNAP when overexpressed. At least in some cases, the isolated DNA binding domain is capable of specifically binding its DNA recognition site.

In many instances, interaction between σ54 RNAP and the activator is enhanced by a cellular factor which promotes DNA bending between the UAS and the σ54 promoter (Freundlich *et al.*, (1992) Mol. Microbiol. 6:2557-2563). This factor, known as integration host factor (IHF) acts to promote transcription from σ54 promoters.

Summary of the Invention

We provide herein a novel screening system which is based on transcriptional activators of σ54-based promoters.

According to a first aspect of the invention, therefore, there is provided a method for detecting a protein-nucleic acid interaction between a acid molecule and a protein molecule, comprising the steps of:

a) providing one or more hybrid σ54 activator proteins comprising a heterologous nucleic acid binding sequence and a constitutively active σ54 transcription activating domain;

b) providing one or more nucleic acid molecules comprising a binding site for the nucleic acid binding sequence and a binding site for σ54 RNAP, which directs the expression of a reporter gene and leads to upregulation thereof in response to activation by the σ54 transcription activating domain; and

c) detecting expression of the reporter gene.

The invention provides a reporter system which is characterised by very low levels of background expression, since the σ54 polymerase is transcriptionally incompetent in the absence of a σ54 transcriptional activator. Since, at physiological concentrations, the binding of the transcriptional activator to the nucleic acid is required in order to activate transcription by σ54 RNAP, the system of the invention may be used as a tool for investigating and/or screening protein/nucleic acid interactions exploiting the reporter gene read-out.

In the first aspect of the invention, either the nucleic acid binding protein or the nucleic acid molecule may be provided in the form of a repertoire of molecules. Repertoires of hybrid σ54 activator proteins preferably are partially or completely randomised at least in the heterologous nucleic acid binding sequence. This allows selection from the library of molecules having desired nucleic acid binding characteristics.

Repertoires of nucleic acid molecules advantageously are partially or completely randomised in the binding site for the nucleic acid binding sequence of the σ54 activator protein. This allows selection of nucleic acid molecules having desired binding sites for the chimeric activators.

In a second aspect of the invention, there is provided a system for selecting protein-protein interactions based on the constitutively active hybrid σ54 activators described above. The system according to the invention is conceptually similar to the yeast two-hybrid system.

Accordingly, there is provided a method for detecting a protein-protein interaction, comprising the steps of:

a) providing a first hybrid protein comprising a nucleic acid binding sequence and a first polypeptide sequence bait;

b) providing a second hybrid protein comprising a prey polypeptide sequence and constitutively active σ54 transcription activating domain;

c) providing a nucleic acid molecule comprising a binding site for the nucleic acid binding sequence and binding site for σ54 RNAP which directs the expression of a

reporter gene and leads to upregulation thereof in response to activation by the σ54 transcription activating domain;

d) incubating the first and second hybrid proteins together with the nucleic acid molecule such that the prey and bait polypeptide sequences may bind, thereby forming a hybrid protein comprising both a nucleic acid binding sequence and a σ54 transcription activating domain; and

e) detecting expression of the reporter gene.

As will be apparent to those skilled in the art, reference to a "binding site" for the nucleic acid binding sequence includes the provision of several appropriately spaced binding sites in the nucleic acid molecule.

As with the yeast two-hybrid system, in which a modular transcription factor is assembled though binding of DNA binding domain/bait and transcription activating domain/prey hybrids, the association of the nucleic acid binding sequence and the σ54 transcription activating domain through the bait/prey interaction allows the detection of, and screening for, protein-protein binding interactions *in vivo* and *in vitro*. Advantageously, the bait and/or prey polypeptide sequences are provided in the form of repertoires, which may be partially or completely randomised. This allows selection of prey polypeptides based on their ability to form interactions with a desired bait (or vice versa). As the assay may be conducted *in vivo*, in a bacterium, the invention permits the detection of *in vivo* binding interactions between polypeptides in bacteria.

It will be apparent that the hybrid proteins useful in the methods of the invention are advantageously provided in the form of nucleic acid vectors or libraries thereof capable of expressing said proteins in a host bacterium. Advantageously, the vector(s) include first and second chimeric genes which encode the hybrid proteins of the invention. Preferably, the vectors also include means for replication in bacteria. Also included may be one or more marker genes, the expression of which in the bacterium permits selection of cells containing the vector(s) from cells that do not contain the vector(s). Preferably, the vector(s) are plasmid(s).

In a third aspect, the invention provides a method for screening a repertoire of candidate DNA-bending polypeptides, comprising the steps of:

a) providing a repertoire of candidate polypeptide factors with potential to induce bending of DNA;

b) providing a σ54 activator protein comprising a nucleic acid binding sequence and a σ54 transcription activating domain;

c) providing a nucleic acid molecule comprising a binding site for the nucleic acid binding sequence and binding site for σ54 RNAP which directs the expression of a reporter gene and leads to upregulation thereof in response to activation by the σ54 transcription activating domain;

d) incubating the repertoire and σ54 activator together with the nucleic acid molecule in a HIF^- host cell, such that σ54 activator and the nucleic acid molecule may interact, and transcription activated from the σ54 RNAP binding site in a manner dependent on DNA bending by the polypeptide factors; and

e) detecting expression of the reporter gene.

It is known that activation by σ54 activators may be regulated by factors which induce DNA bending in the target gene. For example, the host factor IHF is known to potentiate σ54 activation; moreover, it may be replaced by alternative DNA bending polypeptides, or by intrinsically bent DNA.

The invention moreover provides methods for development of improved σ54 activator-based tools.

The first chimeric gene includes a nucleic acid sequence that encodes a nucleic-binding domain and a first (bait) test protein or protein fragment in such a manner that the first test protein is expressed as part of a hybrid protein with the nucleic acid-binding domain.

The second chimeric gene also includes a promoter and a transcription termination signal to direct transcription. The second chimeric gene moreover includes a nucleic acid sequence that encodes a σ54 transcriptional activation domain and a second (prey) test protein or protein fragment into the vector, in such a manner that the second test protein is

capable of being expressed as part of a hybrid protein with the transcriptional activation domain.

The invention moreover provides kits for practising the invention, which kits advantageously comprise a container, two vectors, and a host cell. The first vector contains a promoter and may include a transcription termination signal functionally associated with the first chimeric gene in order to direct the transcription of the first chimeric gene. The chimeric gene advantageously comprises one or more unique restriction site(s) to insert a nucleic acid sequence encoding a test bait polypeptide. The kit also may also include a second vector which contains a second chimeric gene, optionally comprising one or more unique restriction site(s) to insert a nucleic acid sequence encoding the prey polypeptide; alternatively, the second chimeric gene may be present on the same vector as the first chimeric gene.

Brief description of the Figures

Figure 1A is a schematic representation of σ54 RNAP activation by σ54 activator NifA.

Figure 1B is a schematic representation of the invention, in which the σ54 DNA binding domain is replaced with a heterologous GCN4 DNA binding domain..

Figure 2 is a schematic representation of the first aspect of the present invention, in which a library of DNA binding domains is screened together with a library of DNA binding domain binding sites to identify protein:DNA binding pairs.

Figure 3 shows the activation of transcription by NifA-chimera as expressed as percent of wt activity (NifA/UAS). Nif-GCN4 (in presence of the NifAΔC coactivator (NifADC)) show close to wt activity. Equal activity is observed for the two distinct GCN$ DNA recognition sites (ATF/Creb and AP-1). Less than 1% wt activity is observed with a non-cognate reporter such as one bearing the wt nifH UAS.

Figure 4 shows the activation of transcription by NifA-chimera as expressed as percent of wt activity (NifA/UAS). Nif-ERDBD (in presence of the NifAΔC coactivator (NifADC)) shows ca. 80% of wt activity. Very little activation is observed with a non-cognate reporter bearing the DNA recognition site (GRE) for the closely related Glucocorticoid receptor.

Figure 5 shows the coactivation by different NifA variants as expressed as percent of wt activity (NifAwt). NifA from Klebsiella pneumoniae (NifAKp) is superior to al others, even exceeding wt activity (up to 160%). NifAKp with its DNA domain deleted (NifAΔCKp (NIfADCKp)) is almost as active.

Detailed Description of the Invention

Unless defined otherwise. all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in bacterial cell culture. molecular genetics, nucleic acid chemistry, protein chemistry and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. which are incorporated herein by reference), chemical methods, pharmaceutical formulations and delivery and treatment of patients.

A: Nucleic Acids and Proteins

As used herein, "nucleic acid" refers to any natural nucleic acid, including RNA and DNA as well as synthetic nucleic acid comprising modified or synthetic bases, and mixtures of modified or synthetic bases with natural bases. Such modified and/or synthetic bases may be referred to as derivatives of DNA or RNA. Preferably, "nucleic acid" refers to DNA.

The invention includes the use of modified and/or artificial "nucleic acids". A number of modifications have been described that alter the chemistry of the phosphodiester backbone, sugars or heterocyclic base components of nucleic acids.

Among useful changes in the backbone chemistry are phosphorothioates; phosphorodithioates, where both of the non-bridging oxygens are substituted with sulphur; phosphoroamidites; alkyl phosphotriesters and boranophosphates. Achiral phosphate derivatives include 3'-O'-5'-S-phosphorothioate, 3'-S-5'-O-phosphorothioate, 3'-CH2-5'-O-phosphonate and 3'-NH-5'-O-phosphoroamidate. Peptide nucleic acids replace the entire phosphodiester backbone with a peptide linkage.

Sugar modifications are also known. The α-anomer of deoxyribose may be used, where the base is inverted with respect to the natural β-anomer. The 2'-OH of the ribose sugar may be altered to form 2'-O-methyl or 2'-O-allyl sugars, which provides resistance to degradation without comprising affinity.

Modification of the heterocyclic bases must maintain proper base pairing. Some useful substitutions include deoxyuridine for deoxythymidine; 5-methyl-2'-deoxycytidine and 5-bromo-2'-deoxycytidine for deoxycytidine. 5-propynyl-2'-deoxyuridine and 5-propynyl-2'-deoxycytidine have been shown maintain biological activity when substituted for deoxythymidine and deoxycytidine, respectively.

As used herein, the term "protein" includes single-chain polypeptide molecules as well as multiple-polypeptide complexes where individual constituent polypeptides are linked by covalent or non-covalent means. As used herein, the terms "polypeptide" and "peptide" refer to a polymer in which the monomers are amino acids and are joined together through peptide or disulphide bonds. The term domain also refers to polypeptides and peptides having biological function. A peptide useful in the invention will have a binding or transcription activating capability, i.e., with respect to binding to nucleic acids, other proteins or polypeptides, and activation of σ54 RNAP transcription. It also may have another biological function that is a biological function of a protein or domain from which the peptide sequence is derived.

A hybrid protein is a protein or polypeptide which comprises constituent parts derived from at least two naturally-occurring or artificial proteins. In particular, it may comprise the DNA-binding domain of one protein and the protein-binding or transcription activating domain of a second protein.

B: σ54 Activators

Activators of σ54 transcription are well known and have been reviewed, for example, by Buck *et al.*, J Bacteriol. 2000 Aug;182(15):4129-36; Studholme and Buck, FEMS Microbiol Lett. 2000 May 1;186(1):1-9; Shingler, Mol Microbiol. 1996 Feb;19(3):409-16; Goosen and van der Putte, Mol Microbiol. 1995 Apr;16(1):1-7; Merrick, Mol Microbiol. 1993 Dec;10(5):903-9; and others. A family of such activator proteins has been defined, and its members found to share homology in the central (catalytic) domain which is responsible for σ54 RNAP activation.

Members of the family include the following (the numbers are GenBank accession numbers)

dbj|BAA16379.1| (D90877) FORMATE HYDROGENLYASE TRANSCRIPTIONAL ACTIVATOR.
emb|CAA26472.1| (X02616) pot. NifA gene product (aa 1-484) [Klebsiella pneumoniae]
emb|CAA53584.1| (X75972) anfA [Rhodobacter capsulatus]
emb|CAA92413.1| (Z68203) NifA homologue [Rhizobium sp.]
emb|CAA93242.1| (Z69251) MopR [Acinetobacter calcoaceticus]
emb|CAB53157.1| (X07567) NifA1 [Rhodobacter capsulatus]
emb|CAB56537.1| (AJ249642) response regulator [Pseudomonas stutzeri]
gb|AAA58220.1| (U18997) ORF_o532 [Escherichia coli]
gb|AAA99303.1| (L43064) regulatory protein [Pseudomonas aeruginosa]
gb|AAB91397.1| (AF033203) NifAII protein [Rhodobacter capsulatus]
gb|AAC05586.1| (AF006075) regulatory protein [Bacillus subtilis]
gb|AAC37124.1| (L81176) FleQ [Pseudomonas aeruginosa]

gb|AAC45640.1| (AF010585) putative sigma 54 activator [Caulobacter crescentus]
gb|AAC46367.1| (AF014113) two-component response regulator [Vibrio cholerae]
gb|AAD34591.1|AF145956_1 (AF145956) transcriptional activator NifA [Rhodospirillum rubrum]
gb|AAD38416.1| (AF155934) NifA [Alcaligenes faecalis]
gb|AAF28395.1| (AF069392) FlaM [Vibrio parahaemolyticus]
gb|AAF33506.1| (AF170176) Salmonella typhimurium transcriptional regulatory protein
gb|AAF61932.1| (AF230804) sigma-54 activator protein Act1 [Myxococcus xanthus]
gb|AAF85342.1|AE004061_7 (AE004061) two-component system, regulatory protein [Xylella fastidiosa]
gb|AAF94676.1| (AE004230) sigma-54 dependent response regulator [Vibrio cholerae]
gb|AAF95280.1| (AE004286) sigma-54 dependent response regulator [Vibrio cholerae]
gb|AAF96095.1| (AE004358) sigma-54 dependent transcriptional regulator [Vibrio cholerae]
gb|AAG01527.1|AF288483_1 (AF288483) NifA [Azospirillum brasilense]
pir||A48291 ornithine decarboxylase inhibitor - Escherichia coli
pir||B49940 nitrogen regulator I homolog - Escherichia coli
pir||C70320 transcription regulator NifA family - Aquifex aeolicus
pir||C70396 transcription regulator NtrC family - Aquifex aeolicus
pir||C70454 transcription regulator NtrC family - Aquifex aeolicus
pir||D70315 transcription regulator NtrC family - Aquifex aeolicus
pir||H69581 transcription activator of acetoin dehydrogenase operon acoR - Bacillus subtilis
pir||I39719 nitrogen regulatory protein - Agrobacterium tumefaciens
pir||JC5471 regulatory protein NifA - Azospirillum lipoferum
pir||T08624 probable NtrC-type response regulator - Eubacterium acidaminophilum
sp|P03027|NIFA_KLEPN NIF-SPECIFIC REGULATORY PROTEIN
sp|P09570|NIFA_AZOVI NIF-SPECIFIC REGULATORY PROTEIN
sp|P12627|VNFA_AZOVI NITROGEN FIXATION PROTEIN VNFA
sp|P14375|HYDG_ECOLI TRANSCRIPTIONAL REGULATORY PROTEIN HYDG
sp|P21712|YFHA_ECOLI HYPOTHETICAL 49.1 KD PROTEIN IN GLNB-PURL INTERGENIC REGION (ORFXB)

sp|P24426|NIFA_RHILT NIF-SPECIFIC REGULATORY PROTEIN
sp|P25852|HYDG_SALTY TRANSCRIPTIONAL REGULATORY PROTEIN HYDG
sp|P27713|NIFA_HERSE NIF-SPECIFIC REGULATORY PROTEIN
sp|P30667|NIFA_AZOBR NIF-SPECIFIC REGULATORY PROTEIN
sp|P38035|RTCR_ECOLI TRANSCRIPTIONAL REGULATORY PROTEIN RTCR
sp|P54929|NIFA_AZOLI NIF-SPECIFIC REGULATORY PROTEIN
sp|P56266|NIFA_KLEOX NIF-SPECIFIC REGULATORY PROTEIN
sp|Q06065|ATOC_ECOLI ACETOACETATE METABOLISM REGULATORY PROTEIN ATOC (ORNITHINE/ARGININE
sp|Q46802|YGEV_ECOLI HYPOTHETICAL SIGMA-54-DEPENDENT TRANSCRIPTIONAL REGULATOR IN
sp|Q53206|NIFA_RHISN NIF-SPECIFIC REGULATORY PROTEIN
sp|Q9ZIB7|TYRR_ERWHE TRANSCRIPTIONAL REGULATORY PROTEIN TYRR

Moreover, a number of polypeptides belonging to the σ54 activator family have been described whose 3D structures are known. These include: 113161 acetoin catabolism regulatory protein; 113629 alginate biosynthesis transcriptional regulatory protein ALGB; 266789 type 4 fimbriae expression regulatory protein PILR; 113833 nitrogen fixation protein ANFA; 138884 nitrogen fixation protein VNFA; 128219 nif-specific regulatory protein; 3024194 nif-specific regulatory protein; acetoacetate metabolism regulatory protein ATOC 1168553 (ornithine/arginine decarboxylase inhibitor) (ornithine decarboxylase antizyme); 417166 transcriptional regulatory protein HYGD; 266622 nif-specific regulatory protein; 1352500 nif-specific regulatory protein; 128224 nif-specific regulatory protein; 128225 nif-specific regulatory protein; 128221 nif-specific regulatory protein; 128226 nif-specific regulatory protein; 1346014 transcriptional regulatory protein FLBD; 549560 hypothetical sigma-54-dependent transcriptional regulator in GUTQ-HYPF intergenic region; 139857 transcriptional regulatory protein XYLR (67 kd protein); 120053 formate hydrogenlyase transcriptional activator; 2507375 hypothetical 49.1 kd protein in GLNB-PUR1 intergenic region (ORFXB) (orf-2); 134961 signal-transduction and transcriptional-control protein; 1171795 nitrogen assimilation regulatory protein; 417388 nitrogen regulation protein nr(i); 123466 hydrogenase transcriptional regulatory protein HOXA; 399925 hydrogenase transcriptional regulatory protein HOXA; 585586 nitrogen

assimilation regulatory protein NTRX; 118399 c4-dicarboxylate transport transcriptional regulatory protein DCTD; 585267 pathogenicity locus probable regulatory protein HRPR; 1346313 pathogenicity locus probable regulatory protein HRPS; 549447 pathogenicity locus probable regulatory protein WTSA; 585909 arginine utilization regulatory protein ROCR; 136600 transcriptional regulatory protein TYRR; 1174836 transcriptional regulatory protein TYRR homolog; 123748 hydrogenase transcriptional regulatory protein HUPR1; 128604 nitrogen regulation protein NTRC; 1169293 glycerol metabolism operon regulatory protein; and 129957 phosphoglycerate transport system transcriptional regulatory protein PGTA . The numbers are GenBank gi numbers.

Preferably, the hybrid σ54 activator is based on the NifA activator. The Nif family of bacterial enhancers regulate expression of nitrogenase components from σ54 promoters in nitrogen-fixing bacteria, and are inhibited by NifL (Austin S. et al (1994) *J. Bacteriol.* 176, 3460). In bacteria lacking NifL, NifA is constitutively active. NifA is modular in architecture and it is shown herein that this allows for the swapping of the natural DNA-binding domain (DBD) for heterologous DBDs. Such NifA-DBD chimaeras are inactive on the wild type promoter, but activate transcription from hybrid promoters bearing their cognate target sequences.

Advantageously, the hybrid σ54 activator may be based on *E. coli* PspF (see Jovanovic *et al.*, (1996) J. Bacteriol. 178:1936-1945). PspF lacks the N-terminal regulatory domain typical of σ54 activators, and is constitutively active but negatively regulated by PspA. Thus, in bacteria lacking PspA, PspF is constitutively active.

Other σ54 activators may be rendered constitutively active by removal of the N-terminal regulatory domain or by appropriate mutation.

Nucleic acid binding sequences or domains are known in the art and may be derived from σ54 activator proteins or any other DNA binding proteins, whether naturally-occurring or synthetic. Moreover, DNA-binding domains may be synthesised by partial or complete randomisation. Many naturally-occurring DNA-binding proteins contain independently folded domains for the recognition of DNA, and these domains in turn belong to a large

number of structural families, such as the leucine zipper, the homeodomain, the "helix-turn-helix", the zinc finger and various other transcription factor families.

C: Libraries

The term library refers to a mixture of heterogeneous polypeptides or nucleic acids. The library is composed of members, which have a unique polypeptide or nucleic acid sequence. To this extent, *library* is synonymous with *repertoire*, although in general the term "library" is used herein to denote the source of the repertoire – e.g. a library of nucleic acid molecules which encodes a repertoire of polypeptides. Sequence differences between library members are responsible for the diversity present in the library. The library may take the form of a simple mixture of polypeptides or nucleic acids, or may be in the form organisms or cells, for example bacteria, viruses, animal or plant cells and the like, transformed with a library of nucleic acids. Advantageously, the nucleic acids are incorporated into expression vectors, in order to allow expression of the polypeptides encoded by the nucleic acids. In a preferred aspect, therefore, a library may take the form of a population of host organisms, each organism containing one or more copies of an expression vector containing a single member of the library in nucleic acid form which can be expressed to produce its corresponding polypeptide member. Thus, the population of host organisms has the potential to encode a large repertoire of genetically diverse polypeptide variants.

Libraries of hybrid proteins may be prepared and selected together with libraries of hybrid nucleic acids. "Crossing" of hybrid libraries is performed by combinatorial infection, which has been employed successfully to generate very large antibody libraries (Griffiths et al (1994) EMBO J. 13, 3245).

Although libraries for use in the present invention may be phage libraries, as is known in the art, it is possible to use alternative libraries which are constructed using other vectors, such as plasmids. In any case, the present invention does not require the library to be capable of "display" of the gene product at the bacterial surface, as with phage libraries;

rather, the gene product is preferable expressed intracellularly, and is advantageously not expressed as a fusion with a vector gene product.

DNA binding domain libraries are preferably based on a known DNA binding domain architectures (e.g. basic leucine zipper, bZIP) and may be derived using PCR amplification with "family-specific" primers. Such libraries may be crossed with hybrid-promoters bearing defined target sequences or libraries of target sequences. In addition to providing information on the distribution of members of the family in a given genome, such libraries may be used to identify and study proteins or molecular compounds that modify DNA interaction within a family of DNA binding domains, for example Tax (from HTLV-1) in the case of bZIP proteins.

In an alternative embodiment, they may also be used to select DNA binding domains which conditionally bind their target sequence only in the presence of other factors such as protein cofactors or small molecular compounds, for example drugs that intercalate into DNA or alter the degree of supercoiling or recognise DNA sequences which have been modified chemically (e.g. methylated). The system can also be used "in reverse" i.e. to select proteins or molecular compounds that disrupt a particular DNA-protein interaction or to select DNA binding domains that do not bind a particular target sequence or library thereof.

More advanced libraries are preferably derived directly from genomic DNA or cDNA libraries and selected on hybrid promoters bearing a repertoire of target sequences, comprising either a stretch of randomised sequence or a library of inserts derived from fragmented genomic DNA. Data obtained in this way allows the compilation of a genomic directory of DNA binding domains and the building of a promoter-DNA binding domain interaction map.

D: Hybrid polypeptides

The generation of hybrid polypeptides by domain fusion is well known in the art and may be effected by fusing polypeptides or, preferably, by fusing nucleic acids which encode

the polypeptides. It has been known since 1976 that DNA binding and transcriptional activator domains are separable. and can be swapped between proteins; see Ma and Ptashne, who reported (Cell, (1987) 51, 113-119; Cell. (1988)55, 443-446) that when both the GAL4 N-terminal domain and C-terminal domain are fused together in the same protein, transcriptional activity is induced. Other proteins are also known function as transcriptional activators via the same mechanism. For example, the GCN4 protein of Saccharomyces cerevisiae as reported by Hope and Struhl, Cell. 46, 885-894 (1986), the ADR1 protein of Saccharomyces cerevisiae as reported by Thukral et al., Molecular and Cellular Biology, 9, 2360-2369, (1989) and the human estrogen receptor, as discussed by Kumar et al., Cell, 51, 941-951 (1987) both contain separable domains for DNA binding and for maximal transcriptional activation.

The same is specifically known of the σ54 bacterial transcriptional activators, although a genetic screen based thereon has not been proposed. Therefore. the present invention may be carried out using techniques which are known to those skilled in the art, particularly as applied to 2-hybrid techniques in eukaryotic cells.

Synthesis of chimeric genes for the purposes of the present invention may be carried out by any desired means, including polynucleotide synthesis and mutagenesis approaches. For example, a number of methods for site-directed mutagenesis are known in the art, from methods employing single-stranded phage such as M13 to PCR-based techniques (see "PCR Protocols: A guide to methods and applications", M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White (eds.). Academic Press, New York, 1990). Preferably, the commercially available Altered Site II Mutagenesis System (Promega) may be employed, according to the directions given by the manufacturer.

E: Host Cells

Host cells useful in conjunction with the present invention are prokaryotic cells, advantageously bacterial cells. *E. coli* is the preferred host; however, host cells may belong to any species or genus in which σ54 RNAP-driven transcription is possible, such as *Klebsiella*, *Rhodobacter*, *Rhizobium*, *Acinetobacter*, *Pseudomonas*, *Escherichia*,

Bacillus, Caulobacter, Vibrio, Rhodospirillum, Alcaligenes, Salmonella, Myxococcus, Xylella, Azospirillum, Aquifex, Agrobacterium and other organisms. In E.coli, the preferred configuration is a modified strain, in which a truncated form of Nif (or another activator) is coexpressed to boost specific activation (see Methods).

Preferably, the host cells lack repressors of the σ54 activator being used, such that the transcription activating domain is constitutively active. Repressors may be deleted by genetic mutation and/or selection, or inhibited by expression of antisense constructs, or the like. In general, due to the accessibility of bacterial genetics, especially in *E. coli*, deletion of repressor genes is straightforward to those skilled in the art.

F: Reporter Genes

Reporter genes of various types are known in the art and may be used in conjunction with the present invention. A "reporter gene", as referred to herein, may be the coding sequence which encodes a detectable gene product, or the coding sequence including the necessary control sequences for its expression in accordance with the invention, as appropriate.

Advantageously, the reporter gene is selected from the group consisting of metabolic markers such as the lac operon (lacZ, lacY and lacA); proteins conferring a fluorescent phenotype, such as GFP; proteins conferring antibiotic resistance, such as Zeo; and proteins conferring another selectable property.

Certain reporters, such as the LacZ gene, are widely used in bacterial genetics and are useful in the performance of the invention. However, other genes may also be employed, including fluorescent proteins. For example, green fluorescent proteins (GFPs) of cnidarians, which act as their energy-transfer acceptors in bioluminescence, can be used in the invention. A green fluorescent protein, as used herein, is a protein that fluoresces green light, and a blue fluorescent protein is a protein that fluoresces blue light. GFPs have been isolated from the Pacific Northwest jellyfish, *Aequorea victoria*, from the sea pansy, *Renilla reniformis*, and from *Phialidium gregarium*. (Ward et al., 1982,

Photochem. Photobiol., 35: 803-808; Levine et al., 1982. Comp. Biochem. Physiol.,72B: 77-85).

A variety of *Aequorea*-related GFPs having useful excitation and emission spectra have been engineered by modifying the amino acid sequence of a naturally-occurring GFP from Aequorea victoria. (Prasher et al., 1992, Gene, 111: 229-233; Heim et al., 1994, Proc. Natl. Acad. Sci. U.S.A., 91: 12501-12504; PCT/US95/14692). As used herein, a fluorescent protein is an Aequorea-related fluorescent protein if any contiguous sequence of 150 amino acids of the fluorescent protein has at least 85% sequence identity with an amino acid sequence, either contiguous or non-contiguous, from the wild-type Aequorea green fluorescent protein (SwissProt Accession No. P42212). Similarly, the fluorescent protein may be related to *Renilla* or *Phialidium* wild-type fluorescent proteins using the same standards.

Aequorea-related fluorescent proteins include, for example, wild-type (native) *Aequorea victoria* GFP, whose nucleotide and deduced amino acid sequences are presented in GenBank Accession Nos. L29345, M62654, M62653 and others *Aequorea*-related engineered versions of Green Fluorescent Protein, of which some are listed above. Several of these, i.e., P4, P4-3, W7 and W2 fluoresce at a distinctly shorter wavelength than wild type.

A specific advantage of fluorescent proteins is that they facilitate FACS sorting of cells in a manner dependent on reporter gene expression (Norman, S.O. (1980). Flow cytometry. *Med. Phys.* 7, 609-615; Mackenzie, N.M. & Pinder, A.C. (1986). The application of flow microfluorimetry to biomedical research and diagnosis: a review. *Dev. Biol. Stand.* **64**, 181-193).

Other reporter genes may complement auxotrophic mutations, confer antibiotic resistance or other selectable characteristics to the host bacteria. Reporter genes may be wholly or partly heterologous to the host cell, and introduced by mutagenesis and/or transformation with appropriate vectors. Alternatively, endogenous σ54-responsive genes may be used as reporter genes.

The reporter gene also contains a binding site for σ54 RNAP. The consensus sequence for σ54 RNAP binding is 5' TGGCAC-N5-TTGCa/t 3'. This sequence is located at -12 to -24 with respect to the start of transcription, whilst the more common sigma 70 recognition sequence is situated at -10 to/ -35. Both the GG & GC must be on the same face of the DNA helix.

In order to increase specificity, combinations of two or more reporter genes (preferably in tandem) may be used.

Where the reporter gene is chimeric, i.e. comprises heterologous binding sites for the nucleic acid binding sequence and σ54 RNAP binding sites incorporated into the same nucleic acid, the spacing between the σ54 RNAP binding site and the nucleic acid binding sequence binding site is preferably conserved with respect to the natural gene from which the σ54 RNAP binding site is taken. Advantageously, the spacing is at least calculated such that the spatial relationship of the elements on respective faces of the nucleic acid helix is maintained.

Reporter genes advantageously comprise a binding site for a further activation factor, such as IHF. These factors are believed to induce bending of the DNA, thus potentiating activation of σ54 RNAP-driven transcription by σ54 activators. Alternatively, the DNA itself may be intrinsically bent, thus providing constitutive potentiation of σ54-specific activation.

G: Configurations of the Invention

The present invention may be configured in three basic ways: a first configuration, in which reporter gene activation is dependent on the interaction between the nucleic acid and a nucleic acid binding domain on the hybrid protein; a second configuration, in which reporter gene activation is dependent on interaction between bait and prey polypeptides which serves to bring together two or more components of the hybrid protein; and a third configuration, in which reporter gene activation by a σ54 activator is dependent on the

presence of DNA-bending polypeptides. As referred to herein, an interaction is advantageously a binding interaction.

Where the invention is configured to detect protein-nucleic acid interaction, libraries of proteins and/or nucleic acids may be prepared as described above. Proteins having improved nucleic acid binding, or nucleic acid sequences having improved affinity for protein domains, may be developed by mutagenesis and selection of candidate sequences. Alternatively, protein and/or nucleic acid sequences may be used to identify, *in vivo*, cognate binding partners.

Chimeric σ54 activators also offer the opportunity to better understand aspects of the process of transcriptional activation at σ54 promoters. In the case of NifA, t is known that binding of the target sequence together with ATP binding promotes oligomerisation of NifA. It is believed that it is the oligomer which contacts the polymerase and catalyses the ATP-driven isomerisation of the polymerase holoenzyme. Taking advantage of the superactivation effect described above it may be possible to address questions such as which components of the oligomer (e.g. the DNA-bound NifA vs. NifAΔC, i.e. NifA with the DNA binding domain removed), which are contacting the polymerase and/or coupling ATP hydrolysis to transcriptional activation etc. Furthermore, usage of NifAΔC cofactors from different species (together with their diversification by PCR shuffling) allows identification of the sequence regions critical for transcriptional activation and a "maturation" of the NifAΔC coactivator. Indeed, we have found the NifA from *K. pneumoniae* to be a superior cofactor to *A. vinelandii* NifA. Finally, it may be possible to use chimera of a known DNA binding domains (e.g. GCN4) and a cDNA library as a prokaryotic "enhancer" trap, to isolate σ54 activators on a genome-wide scale.

Configuration of the invention to detect protein-protein interactions follows the general scheme of the yeast two-hybrid assay, and the reagents used in the invention may be set up accordingly. In general, therefore, the invention will comprise a nucleic acid binding domain-bait fusion, and a prey-σ54 activator domain fusion. Although, in general, "bait" refers to a known polypeptide and "prey" to an unknown polypeptide, the terms may be

used interchangeably. Indeed, the invention comprises configurations in which both bait and prey are known, or both are unknown.

Binding between the bait and the prey result in constitution of a hybrid protein which comprises both a nucleic acid binding domain and a σ54 activator domain. The hybrid protein is able to activate transcription from a reporter gene, thus providing a bait:prey binding-dependent signal.

Protein-protein interactions may be selected using the preferred NifA system, in which the hybrid σ54 transcriptional activator includes the NifA activation domain. The NifA bacterial two-hybrid system may be used for the generation of interaction matrices between cDNA libraries. Ultimately such interaction matrices may yield an interaction map of the proteins of an organism. The invention provides an alternative to the yeast two hybrid system.

Systems based on σ54 have a number of advantages over the other systems that are available, e.g. the conceptually similar yeast one and two-hybrid system. A bacterial host allows substantially larger repertoires to be obtained and thus a much larger molecular diversity to be screened. In particular, using combinatorial infection, the system of the invention allows the "crossing" of both σ54-chimera repertoires with libraries of hybrid reporter constructs, thus permitting coevolution of DNA binding domains, and recognition sites, or coselection of DNA binding domains and target sites from genomic libraries.

Because selection in the σ54-based system is based on a positive readout, i.e. activation of transcription, it is less prone to false positives than other approaches relying on the inhibitory effect of the expressed DNA binding domains, like the transcription interference assay (Elledge S.J. et al (1989) *Proc. Nat. Acad. Sci USA* 86, 3689). In vivo selection in general may result in the selection of novel DNA binding domains that are more attuned to working under realistic conditions, including supercoiling of the recognition site, presence of a large excess of chromosomal DNA and high protein concentration. Another advantage of the system of the invention is that extremely low levels of the hybrid protein

appear to be sufficient to affect maximum activation of transcription. This is particularly helpful in the case of DNA binding domains that are prone to aggregation.

The σ54-based systems of the present invention may be further adapted to take into account potential disadvantages of bacterial expression. For instance, E. coli expression may be suboptimal for large eukaryotic transcription factors. However, large eukaryotic proteins can often be split into smaller domains which retain function and are usually readily expressed in E. coli.

According to the third configuration, a constitutively active σ54 activator may be used to screen a library of candidate DNA-bending polypeptides, preferably in a HIF negative host. Since the degree of activation by the σ54 activator may be dependent on DNA bending by additional factors, the levels of expression of the reporter gene will be modulated by the DNA-bending activity of the candidate DNA-bending polypeptides.

The invention is further described, for the purposes of illustration only, in the following examples.

Examples

NifA from *A. vinelandii* is a well-studied member of the family of bacterial enhancers and it is a positive regulator of the expression of nitrogenase components in diazotrophs. It is inhibited by NifL in response to the presence of oxygen or ammonia. When expressed in *E. coli*, which lacks endogenous NifL or an equivalent, NifA is constitutively active. Because of the highly conserved nature of the activation mechanism of σ54 RNA polymerase, NifA is a very strong activator of transcription in *E. coli*.

Like other members of the family of bacterial enhancer proteins, NifA is modular in architecture, both structurally and functionally, comprising 3 domains, a N-terminal sensor domain , a central activation domain (AD), and a C-terminal DNA binding domain (DBD). The central activation domain (AD) can activate transcription independent of DNA

binding if overexpressed. Thus the DBDs function appears to be primarily to increase the Activator domain's concentration in the promoter proximity.

We have exploited the modularity of the enhancer structure and swapped the natural NifA DNA binding domain (DBD) for heterologous DBDs and libraries thereof. Here we describe the activity of these NifA-chimeras in the activation of transcription from the σ54 dependent promoter nifH and hybrids thereof.

Materials & Methods

Media & Reagents

2xTY, MacConkey agar are described elsewhere (Miller J.H. (1972) *Experiments in molecular genetics*, Cold Spring Harbour, NY). Antibiotics were used at the following concentrations: Ampicillin 0.1 mg/ml, Chloramphenicol 10μg/ml, Streptomycin 25μg/ml. Min-lac medium was essentially M9 medium (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y) supplemented with 1mM $MgSO_4$, 20μM $CaCl_2$, 2% (w/v) lactose, 2mg/ml casamino acids, 40μg/ml L-tryptophan, 5μg/ml thiamine and appropriate antibiotics. Min-lacX plates where essentially M9 plates supplemented 2% lactose, appropriate antibiotics and 40μg/ml X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside).

Strains

TG1ΔK was derived from TG1 (Gibson T. J. (1984) *Studies on the Epstein-Barr virus genome*, University of Cambridge) using the genome integration strategy of Haldimann A. *et al.*, (1996) *Proc. Nat. Acad. Sci USA* 93, 14361. Briefly, NifA (*K. pneumoniae*) residues 1-462 was amplified using Pfu polymerase (Stratagene) and primers 1 (5'- GAG TCA CTA ACG CAT ATG ATC CAT AAA TCC GAT TCG GAC -3'), 2 (5'- CGC GGA TCC AAG CGG CCG CTC ATT AGC GAT GGT TGA ACA GAA TCA C -3') cut with NdeI and BamHI and cloned into the genome targeting suicide vector pSK50D-uidA2 (Haldimann, *Op. Cit.*) and transformed into the Pir^+ host strain BW23473 (Metcalf W.W. et al (1994) *Plasmid* 35, 1). Vectors were isolated and transformed into the Pir^- strain TG1 harbouring the plasmid pINT-ts (Hasan N. et al (1994) *Gene* 150, 51). Chromosomal

integration was induced by a temperature shift to 42°C, which leads to expression of λ integrase from pINT-ts and simultaneously stops its replication. Integrants where identified by Kanamycin resistance and screened for Nif coactivation. Once obtained TG1ΔK was grown routinely without antibiotic selection.

Constructs

Chimeric constructs were based on pDB737 (Austin S. et al (1994) *J. Bacteriol.* 176, 3460 Buck M. et al (1986) *Nature* 320, 374) encoding NifA (*A. vinelandii*) under the control of the T7 promoter in the plasmid pT7-7 (Tabor S. & Richardson C.C. (1985) *Proc Natl Acad Sci USA* 82, 1074). Expression was by leakiness of the T7 promoter. Chimeras were constructed taking advantage of an unique BanII cutting site, in the linker region between the central domain of NifA and the DBD. GCN4 was amplified using Pfu polymerase (Stratagene) and primers 3 (5'- GCT GCC AGC GAG AGC CCG CCG CTC GCC GCG ATT GTG CCC GAA TCC AGT GAT CCT -3') and 4 (5'- GAG CTA AAG CTT TTA TTA GCG TTC GCC AAC TAA TTT CTT TAA TCT GGC -3') cut with BanII and Hind3 and ligated into pDB737 cut with BanII and Hind3. ERDBD was amplified using primers 5 (5'- GTC GAC AAC GAG AGC CCG CCG CTC GCC GCG GAA ACG CGT TAC TGC GCT GTT -3') TGC and 6 (5'- GGT CAG CGC GTG GAT CCT TAA CCA CCA CGA CGG TCT TTA CG-3') cut with BanII and BamHI and ligated into pDB737 cut with BanII and BamHI. The vector p737S1 is derived from pDB737 by replacing the bla gene with aadA conferring streptomycin resistance and the insertion of a fl phage origin for packaging of the vector into filamentous phage particles. Briefly, aadA was amplified using primers 7 (5'- TCA GCG CAC GCT GAC GTC GTG GAA ACG GAT GAA GGC ACG AAC -3'), 8 (5'-CCG CCT GGA GGT GGC CAT TAT TTG CCG ACT ACC TTG GTG ATC TCG CC -3') and cut with AatII and MscI and ligated with pDB737 cut with AatII and ScaI. The resulting vector p737S was cut with AatII, ClaI. The fl ori was amplified using primers 9 (5'- GCT GCC GAC TCG ATC GAT GAA TGG CGA ATG GCG CCT GAT GCG G -3'), 10 (5'-CCG GGT CGT GAC GTC AGT GTT GGC GGG TGT CGG GGC TGG C -3') cut with AatII, ClaI and cloned into the cut p737S to give p737S1. NifA-X chimera were transferred from pDB737 to p737S1 by digestion with NdeI, Hind3 (BamHI for NifA-ERDBD).

Reporter constructs were derived from pACYC184 and the vector pMB1 (Buck M. *et al.* (1986) *Nature* 320, 374). Briefly the lac-operon (lacZYA) was amplified with primers 11 (5'- GAG TCA ATT CGG GGA TCC CGT CGT TTT ACA ACG TCG TGA CTG G-3'), 12 (5'- GAG TCA TTC TGG CCA GTC GAC CGC TCT GCC GGT GGT TAC -3') and cut with BamHI and MscI. The nifH promoter segment from pMB1 was amplified with primers 13 (5'- GAG TCA TTC AAG CTT GCG TGG AAT AAG ACA CAG GGG GCG-3'), 14 (5'- GAG TCA TTC GGG ATC CCC GGA TTT ACC GAT ACC GCC TTT ACC -3') and cut with Hind3, BamHI and the 2 fragments simultaneously ligated with pACYC184 cut with Hind3 and BsaAI to give pMB3. The f1 ori was amplified with primers 15 (5'- GCT GCC GAC TCG GCT AGC GAA TGG CGA ATG GCG CCT GAT GCG G -3'), 16 (GCC GGG TCG CTT TAA AGT GTT GGC GGG TGT CGG GGC TGG C -3') and cut with NheI and DraI and ligated into pMB3 cut with both NheI, XmnI to give pMB31.

Selection and screening

Cells were cotransformed either by simultaneous or sequential electroporation with an expressor construct and a reporter construct and grown overnight with appropriate antibiotic selection at 34^{o}C in M9-lac medium and plated out. β-gal expression was scored either on MacConkey or Minlac-X-gal indicator plates or by ONPG enzyme assay of selected colonies (see below).

Enzyme assay

ONPG assays used to measure β-gal activity were essentially as described by Kolmar H. et al. (1995) *EMBO J* 14, 3895. Briefly, 20μl of an overnight culture is transferred to a microtitre well and 100μl of chloroform saturated Z-buffer (100mM $NaHPO_4$, 1mM KCL, 1mM $MgSO_4$, 50mM β-mercaptoethanol, pH 7.0 (Miller J.H. (1972) *Experiments in molecular genetics*, Cold Spring Harbour, NY) was added and the optical density at 600nm determined using an ELISA reader. Cells were lysed by addition of 50μl Z-buffer with 0.4% (w/v) SDS and incubated at 30^{o}C for 10 min. 50μl of Z-buffer with 4mg/ml O-nitrophenyl-β-D-galactopyranoside were added and the optical density at 420nM was

recorded automatically every 15s over a period of 60min. Specific β-gal activity was calculated from the V_{max} as in Miller (*Op. Cit.*).

Example 1: NifA chimera with heterologous DNA binding domains activate transcription but only from promoters with a cognate recognition site

To investigate in what way transcription activation by NifA was dependent on the NifA DNA binding domain (DBD) and on native nif promoter structure, we prepared NifA-chimeras in which NifA DNA binding domain (DBD) had been replaced by heterologous DBDs of diverse structural architectures. Initially we explored DBDs which, like the NifA wild type (wt) DBD bind to symmetrical DNA recognition sequences such as the basic leucine zipper (bZIP) DBD of the yeast transcription factor GCN4, the Zn-finger domain of the human estrogen receptor DNA binding domain (ERDBD) and determined their capacity to activate transcription of a lacZ reporter gene in vivo from a hybrid nifH promoter, in which the NifA UAS had been deleted and replaced by recognition sites for the heterologous DBDs.

In order to simplify comparison of transcription activation by NifA chimeras with activation by wt NifA, all reporter constructs had a single DNA recognition site. The wt nifH promoter UAS contains three bona fide NifA recognition sites. Deletion of the two sites more distal to the promoter, however, did not appear to reduce transcription activation in our reporter under conditions tested.

Transcription activation by NifA-chimeras was specific in that they only activated lacZ expression from hybrid-promoters bearing their cognate recognition sequences but not from control reporter constructs bearing wild type UAS or a non-cognate site (Fig. 3). In analogy to wt NifA the presence of two or more recognition sites (in phase, see below) did not increase activation by the Nif-GCN4 chimera.

Activity was also dependent on the phasing of the recognition site with respect to the promoter: when the symmetric ATF/CREB recognition site for GCN4 was offset in

increments of 1 bp, optimal activity was observed when the ATF/CREB was centred on the same bp as the symmetric wt UAS. Presumably efficient contact with the RNA Pol holoenzyme requires that the activator be bound on the right face of the DNA.

Transcription activation by NifA-chimeras appears to preserve fine specificity of isolated DBDs. Wild-type GCN4 binds with equal affinity to the symmetric ATF/CREB site as well as to the pseudo-symmetric AP-1 site. Indeed, the NifA-GCN4 chimera showed identical levels of transcription activation in reporter constructs with either of these sites (Fig. 3). A NifA-ERDBD chimera showed strong activity on a reporter with its cognate ERE site but no activity above background levels with reporters bearing the similar GRE recognition site for the closely related glucocorticoid receptor DBD (Fig. 4).

Example 2: Coexpression of wild-type NifA with NifA-chimeras boosts specific transcription activation by NifA chimeras in a specific and DNA independent manner

The level of transcription activation by the NifA-GCN4 and NifA-ERDBD chimeras was lower (ca. 10%) than for wt NifA. However, near wt levels of activity (up to 80%) were reached when wt NifA was coexpressed within the same cell as a "coactivator".

The coactivation was independent of DNA binding, as NifA variants in which the DBD had been deleted (NifAΔC) was found to be just as active as wt NifA. On the other coexpression of an isolated NifA central domain (both the DBD as well as the N-terminal sensor domain deleted (NifAΔNC)) failed to coactivate. NifA derived from different species showed greatly variable efficiencies as coactivators. NifA variants from *K. pneumoniae* (NifA Kp, NifAΔC Kp) were almost three times as effective as NifA, while NifA variants from Rhizobium (NifA Rh1, NifA Rh2) were poorly active as coactivators (Fig. 5).

The coactivator effect was found to enhance only specific transcriptional activation and not background levels of transcription from promoters with non-cognate recognition sites. We therefore constructed an *E. coli* strain, expressing NifAΔC Kp (the *K. pneumoniae* NifA with its DBD deleted) from a weak promoter (phoB) from the chromosome (TG1:ΔK).

The coactivation effect has analogies in eukaryotic transcription. for example the enhancer Sp1, in which isolated Sp1 activation domains can stimulate transcriptional activation by the DNA binding-form of Sp1. a phenomenon termed "superactivation".

Example 3: Tethering of NifA chimera at the UAS is sufficient for activation. but strong activation requires correct positioning

We also investigated transcription activation by NifA-chimeras with asymmetrical recognition sites such as the classic Zn-finger Zif268 as well as the DBD from p53.

Both NifA-Zif268 and NifA-p53 chimeras activated transcription. but only at low levels (2 - 5-fold above the background). However, when the Zif recognition site was duplicated, to give a symmetric palindromic site transcription activation increased substantially. Non-palindromic duplication of the recognition site in tandem did not increase activation.

Thus while simple tethering is sufficient for some activation. only bipartite binding appears to give a strong activation. Presumably, tethering only leads to an approximate positioning of the activation domain with respect to the RNA polymerase holoenzyme, thereby reducing the likelihood of a productive interaction.

Example 4: Selection of active NifA-chimeras by lac complementation

Using expression of the lac operon (lacZYA) from our reporter construct as the read-out of transcription activation allows the selection of active NifA-chimera on the basis of metabolic complementation of a Δlac strain, with lactose as the only carbon source. Initially we spiked populations of NifA-ERDBD with NifA-GCN4 at the ratios $1/10^4$, $1/10^6$ in the presence of the GCN4 cognate reporter ATF/CREB-nifH and grew populations overnight in minimal medium supplied with lactose. Pre- and post selection populations were scored by plating on MacConkey-lactose plates as well as by PCR

screening. The results are summarised in Table 1. Selection factors of up to 10,000 -fold per round were observed. .

Table 1:Selection factors for Nif selection by lac complementation

NifGCN4/NifERDBD	Selection factor
$1/10^4$	40 fold
$1/10^5$	40 fold
$1/10^6$	200 fold
$1/10^7$	4000 fold

Example 5: Selection of active NifA-chimeras by flow cytometry.

Expression of β-galactosidase (lacZ) as the read-out of transcription activation allows the selection of active NifA-chimera on the basis of metabolic complementation of a Δlac strain, grown on lactose as the only carbon source. However, metabolic selection predisposes the system to the generation of false positives. Presumably, the prolonged growth under metabolic selection selects for mutant promoters, active in the absence of a cognate enhancer.

We have observed that that this only occurs for library sizes exceeding 10^6. Indeed, others have found (using a related bacterial two-hybrid system) that it is not possible to retrieve positive clones from dilutions higher than $1/10^6$ by metabolic lac selection (G. Karimova, et al., (1998) Proc Natl Acad Sci USA 95, 12532-7). As it is well known that bacteria can develop a mutator phenotype under adaptive stress (P. D. Sniegowski, P. J. Gerrish, R. E. Lenski, (1997) *Nature* 387, 703-5), we conclude that it is preferable to separate the selection from the amplification (growth) step in order to reduce the likelihood of revertants.

We thus replaced lacZ with the *Aequorea victoria* green fluorescent protein (EGFP. F64L, S65T. ex488 nm. em527nm (the Clontech variant pGFPmut3.1. S65E . S72A. ex501nm. em511nm FACS optimised variant was also tried. but found inferior) as the reporter gene. GFP has the advantage that cells can be grown first and then separated on the basis of fluorescence using fluorescence activated cell sorting (FACS).

We prepared a trial library of mutant GCN4 bZIP DBDs (library size10^8) in which 5 key residues (Asn235, Ala238. Ala239. Ser242. Arg243) of GCN4 interacting with DNA were randomised and selected it against a GFP hybrid reporter with the cognate ATF/CREB site. Library populations were grown overnight at 34°C in non-fluorescent medium NFM (minimal medium supplied with 2%glucose. 0.2% casaminoacids.12 ng/ml L-Trp). For FACS (Cytomation Mofo, 488 nm Laser. FL-1 530/40 filter) an 1 ml aliquot was diluted 10X in NFM and the top 1% fluorescent cell population was sorted into a 96 well plate at 1 cell per well, and grown up overnight at 34°C. Cell fluorescence of the grown up clones was measured by using a SPECTRAmax^{R}GEMINI Dual-Scanning Microplate Spectrofluorometer (Molecular Devices), ex480, em520, (cut-off 515 nm). Plasmids from fluorescent wells were sequenced afterwards. Pre- and post selection populations were also scored by PCR screening as well as by plating on min glu (M9 Minimal medium + glucose) plates and visualised using fluorescence microscope.

10^5 cells were sorted in total, from which 219 cells were in the top 1% fluorescent population and 132 of which were captured to the 96-well plates. 13 cells from these were fluorescent. Selected positives were checked by separating the mutant GCN4-bZIP DBD expressor plasmids. and re-transforming them together with cognate and non-cognate reporter plasmids. None of the selected positives gave a fluorescent signal when combined non-cognate reporter plasmids, but all were fluorescent when combined with the ATF/Creb cognate reporter plasmid (which did not produce any fluorescence when transformed on its own).

This indicates that GFP selection indeed avoids the isolation of false positives. Furthermore, when the library was checked prior to FACS sorting no fluorescent clones

were identified when plating $>10^7$ cells. 1/10 clones plated post selection were fluorescent, suggesting a selection factor in a single round in excess of 10^6-fold.

All publications mentioned in the above specification are herein incorporated by reference. All database sequences denoted by accession or gi numbers are likewise incorporated by reference.

Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

Claims

1. A method for detecting a protein-nucleic acid interaction between a acid molecule and a protein molecule, comprising the steps of:

a) providing one or more hybrid σ54 activator proteins comprising a heterologous nucleic acid binding sequence and a constitutively active σ54 transcription activating domain;

b) providing one or more nucleic acid molecules comprising a binding site for the nucleic acid binding sequence and a binding site for σ54 RNAP, which directs the expression of a reporter gene and leads to upregulation thereof in response to activation by the σ54 transcription activating domain; and

c) detecting expression of the reporter gene.

2. A method according to claim 1, comprising providing a repertoire of hybrid σ54 activator proteins, said repertoire comprising a plurality of different nucleic acid binding sequences.

3. A method according to claim 1, comprising providing a repertoire of hybrid nucleic acid molecules, said repertoire comprising a plurality of different binding sites for the nucleic acid binding sequence.

4. A method according to claim 1, comprising providing both a repertoire according to claim 2 and a repertoire according to claim 3.

5. A method for detecting a protein-protein interaction, comprising the steps of:

a) providing a first hybrid protein comprising a nucleic acid binding sequence and a first polypeptide sequence bait;

b) providing a second hybrid protein comprising a prey polypeptide sequence and constitutively active σ54 transcription activating domain;

c) providing a nucleic acid molecule comprising a binding site for the nucleic acid binding sequence and binding site for σ54 RNAP which directs the expression of a

reporter gene and leads to upregulation thereof in response to activation by the σ54 transcription activating domain;

d) incubating the first and second hybrid proteins together with the nucleic acid molecule such that the prey and bait polypeptide sequences may bind, thereby forming a hybrid protein comprising both a nucleic acid binding sequence and a σ54 transcription activating domain; and

e) detecting expression of the reporter gene.

6. A method according to claim 5, comprising providing a repertoire of first hybrid proteins, said repertoire comprising a plurality of bait polypeptides.

7. A method according to claim 5, comprising providing a repertoire of second hybrid proteins, said repertoire comprising a plurality of prey polypeptides.

8. A method according to claim 5, comprising providing a repertoire of first hybrid proteins and a repertoire of second hybrid proteins, said repertoires comprising a plurality of bait and prey polypeptides.

9. A method for screening a repertoire of candidate DNA-bending polypeptides, comprising the steps of:

a) providing a repertoire of candidate polypeptide factors with potential to induce bending of DNA;

b) providing a σ54 activator protein comprising a nucleic acid binding sequence and a σ54 transcription activating domain;

c) providing a nucleic acid molecule comprising a binding site for the nucleic acid binding sequence and binding site for σ54 RNAP which directs the expression of a reporter gene and leads to upregulation thereof in response to activation by the σ54 transcription activating domain;

d) incubating the repertoire and σ54 activator together with the nucleic acid molecule in a HIF^- host cell, such that σ54 activator and the nucleic acid molecule may interact, and transcription activated from the σ54 RNAP binding site in a manner dependent on DNA bending by the polypeptide factors; and

e) detecting expression of the reporter gene.

10. A method according to any preceding claim. wherein the polypeptides are obtained by expression within a bacterial host cell.

11. A method according to claim 10. wherein the polypeptides are encoded one or more libraries of nucleic acid vectors.

12. A method according to claim 11, wherein a first library of nucleic acid vectors encodes a first chimeric gene, said gene comprising a nucleic acid sequence that encodes a nucleic-binding domain and a nucleic acid sequence encoding a first (bait) test protein or protein fragment in such a manner that the first test protein is expressed as part of a hybrid protein with the nucleic acid-binding domain.

13. A method according to claim 11, wherein a second library of nucleic acid vectors encodes a second chimeric gene, said gene comprising a nucleic acid sequence that encodes a σ54 transcriptional activation domain and a second (prey) test protein or protein fragment into the vector, in such a manner that the second test protein is capable of being expressed as part of a hybrid protein with the transcriptional activation domain.

14. A method according to any preceding claim, wherein the σ54 transcriptional activator is selected from the group consisting of:
dbj|BAA16379.1| (D90877) FORMATE HYDROGENLYASE TRANSCRIPTIONAL ACTIVATOR;
emb|CAA26472.1| (X02616) pot. Nifa gene product (aa 1-484) [Klebsiella pneumoniae];
emb|CAA53584.1| (X75972) anfa [Rhodobacter capsulatus];
emb|CAA92413.1| (Z68203) nifa homologue [Rhizobium sp.];
emb|CAA93242.1| (Z69251) mopr [Acinetobacter calcoaceticus];
emb|CAB53157.1| (X07567) nifa1 [Rhodobacter capsulatus];
emb|CAB56537.1| (AJ249642) response regulator [Pseudomonas stutzeri];
gb|AAA58220.1| (U18997) ORF_o532 [Escherichia coli];
gb|AAA99303.1| (L43064) regulatory protein [Pseudomonas aeruginosa]:

gb|AAB91397.1| (AF033203) nifaii protein [Rhodobacter capsulatus];
gb|AAC05586.1| (AF006075) regulatory protein [Bacillus subtilis];
gb|AAC37124.1| (L81176) fleq [Pseudomonas aeruginosa];
gb|AAC45640.1| (AF010585) putative sigma 54 activator [Caulobacter crescentus];
gb|AAC46367.1| (AF014113) two-component response regulator [Vibrio cholerae];
gb|AAD34591.1|AF145956_1 (AF145956) transcriptional activator nifa [Rhodospirillum rubrum];
gb|AAD38416.1| (AF155934) nifa [Alcaligenes faecalis];
gb|AAF28395.1| (AF069392) flam [Vibrio parahaemolyticus];
gb|AAF33506.1| (AF170176) Salmonella typhimurium transcriptional regulatory protein;
gb|AAF61932.1| (AF230804) sigma-54 activator protein Act1 [Myxococcus xanthus];
gb|AAF85342.1|AE004061_7 (AE004061) two-component system, regulatory protein [Xylella fastidiosa];
gb|AAF94676.1| (AE004230) sigma-54 dependent response regulator [Vibrio cholerae];
gb|AAF95280.1| (AE004286) sigma-54 dependent response regulator [Vibrio cholerae];
gb|AAF96095.1| (AE004358) sigma-54 dependent transcriptional regulator [Vibrio cholerae];
gb|AAG01527.1|AF288483_1 (AF288483) nifa [Azospirillum brasilense];
pir||A48291 ornithine decarboxylase inhibitor - Escherichia coli;
pir||B49940 nitrogen regulator I homolog - Escherichia coli;
pir||C70320 transcription regulator nifa family - Aquifex aeolicus;
pir||C70396 transcription regulator ntrc family - Aquifex aeolicus;
pir||C70454 transcription regulator ntrc family - Aquifex aeolicus;
pir||D70315 transcription regulator ntrc family - Aquifex aeolicus;
pir||H69581 transcription activator of acetoin dehydrogenase operon acor - Bacillus subtilis;
pir||I39719 nitrogen regulatory protein - Agrobacterium tumefaciens;
pir||JC5471 regulatory protein nifa - Azospirillum lipoferum;
pir||T08624 probable ntrc-type response regulator - Eubacterium acidaminophilum;
sp|P03027|NIFA_KLEPN NIF-SPECIFIC REGULATORY PROTEIN;
sp|P09570|NIFA_AZOVI NIF-SPECIFIC REGULATORY PROTEIN;
sp|P12627|VNFA_AZOVI NITROGEN FIXATION PROTEIN VNFA;

sp|P14375|HYDG_ECOLI TRANSCRIPTIONAL REGULATORY PROTEIN HYDG;
sp|P21712|YFHA_ECOLI HYPOTHETICAL 49.1 KD PROTEIN IN GLNB-PURL INTERGENIC REGION (ORFXB);
sp|P24426|NIFA_RHILT NIF-SPECIFIC REGULATORY PROTEIN;
sp|P25852|HYDG_SALTY TRANSCRIPTIONAL REGULATORY PROTEIN HYDG;
sp|P27713|NIFA_HERSE NIF-SPECIFIC REGULATORY PROTEIN;
sp|P30667|NIFA_AZOBR NIF-SPECIFIC REGULATORY PROTEIN;
sp|P38035|RTCR_ECOLI TRANSCRIPTIONAL REGULATORY PROTEIN RTCR;
sp|P54929|NIFA_AZOLI NIF-SPECIFIC REGULATORY PROTEIN;
sp|P56266|NIFA_KLEOX NIF-SPECIFIC REGULATORY PROTEIN;
sp|Q06065|ATOC_ECOLI ACETOACETATE METABOLISM REGULATORY PROTEIN ATOC (ORNITHINE/ARGININE;
sp|Q46802|YGEV_ECOLI HYPOTHETICAL SIGMA-54-DEPENDENT TRANSCRIPTIONAL REGULATOR IN;
sp|Q53206|NIFA_RHISN NIF-SPECIFIC REGULATORY PROTEIN; and
sp|Q9ZIB7|TYRR_ERWHE TRANSCRIPTIONAL REGULATORY PROTEIN TYRR.

15. A method according to any one of claims 1 to 14, wherein the σ54 transcriptional activator is the NifA transcriptional activator or the PspF transcriptional activator.

16. A method according to any one of claims 1 to 14, wherein the hybrid σ54 transcriptional activator is NifA and activation resulting from NifA-σ54 RNAP interaction is enhanced by the coexpression of wild-type or mutant NifA.

17. A method according to claim 16, wherein the hybrid σ54 transcriptional activator is NifA from *Azotobacter vinelandii*, and the wild-type or mutant NifA is NifA from *Klebsiella pneumoniae*.

18. A method according to any preceding claim, wherein the nucleic acid molecule comprises a binding site for a factor which induces DNA bending.

19. A method according to claim 18, wherein the factor is integration host factor (IHF).

20. A method according to any one of claims 1 to 17, wherein the nucleic acid molecule comprises DNA that is intrinsically bent.

21. A method according to any preceding claim, wherein the nucleic acid molecule comprises a nifH promoter from *A. vinelandii* driving a reporter gene.

22. A method according to any preceding claim, wherein the reporter gene is selected from the group consisting of metabolic markers such as the lac operon (lacZ, lacY and lacA); proteins conferring a fluorescent phenotype, such as GFP; proteins conferring antibiotic resistance, such as Zeo; and proteins conferring another selectable property.

23. A method according to any preceding claim, which is carried out in the presence of a compound which modifies protein-protein or protein-DNA interaction.

24. A method according to claim 22, wherein the compound is selected from the group consisting of molecules which alter the structure of the DNA-binding protein; molecules which alter the structure of DNA; and molecules which modify protein-protein interactions.

25. A method according to any preceding claim, which is carried out *in vivo*.

26. A method according to claim 25, wherein the *in vivo* host is *E. coli*.

27. A method according to any one of claims 1 to 24, which is carried out *in vitro*.

FIGURE 1

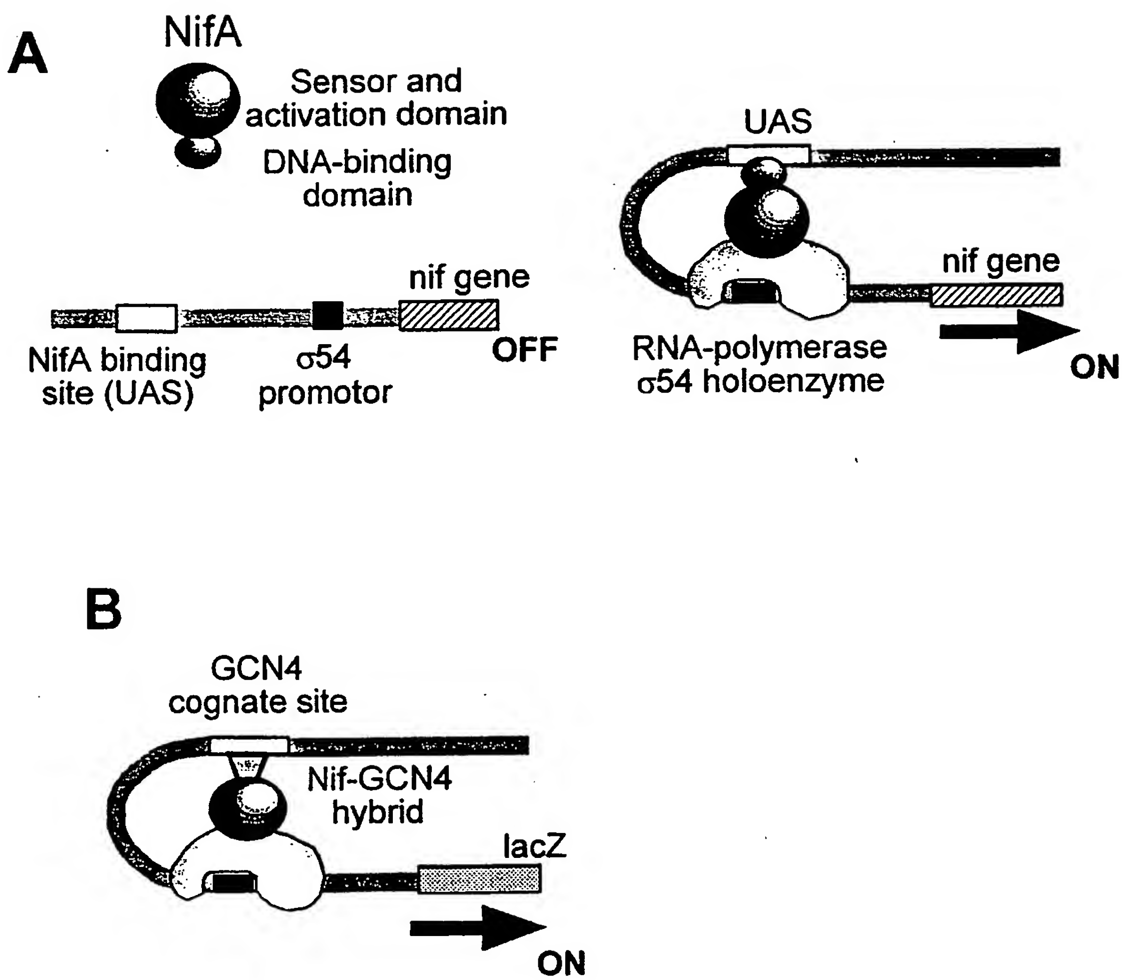

A
NifA
Sensor and activation domain
DNA-binding domain
nif gene
NifA binding site (UAS)
σ54 promotor
OFF
UAS
nif gene
RNA-polymerase σ54 holoenzyme
ON
B
GCN4 cognate site
Nif-GCN4 hybrid
lacZ
ON

FIGURE 2

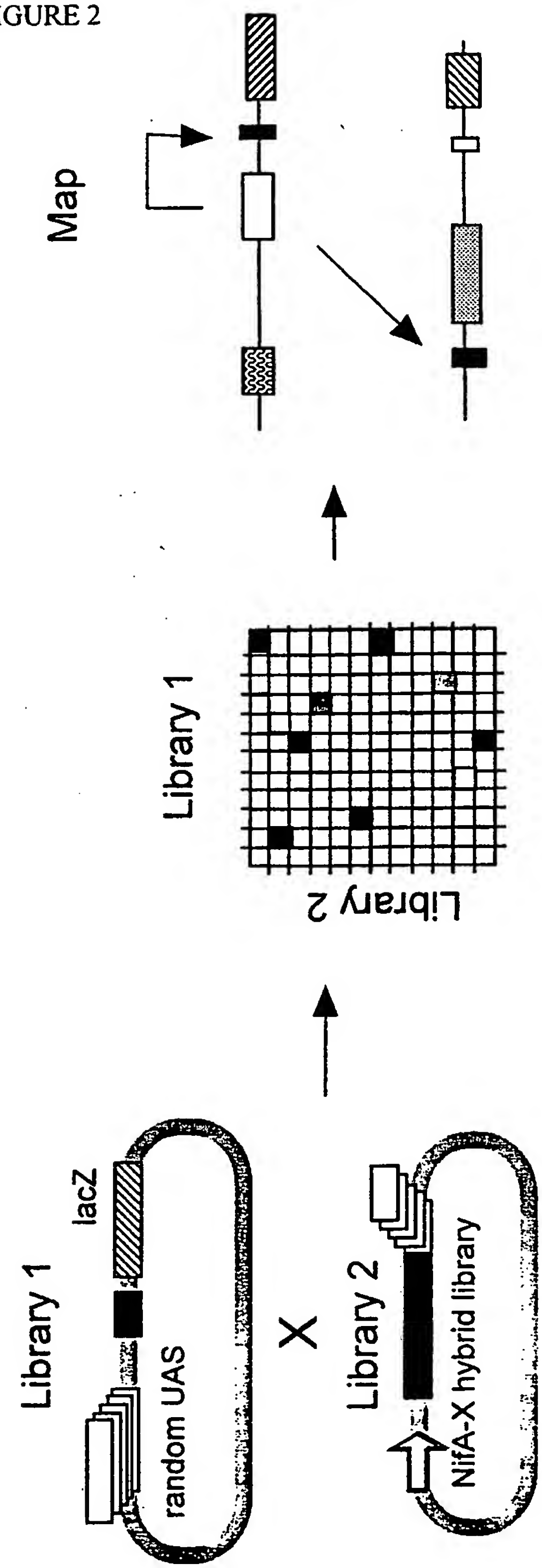

Map
Library 1
Library 2
Library 1
lacZ
random UAS
X
Library 2
NifA-X hybrid library

Figure 3

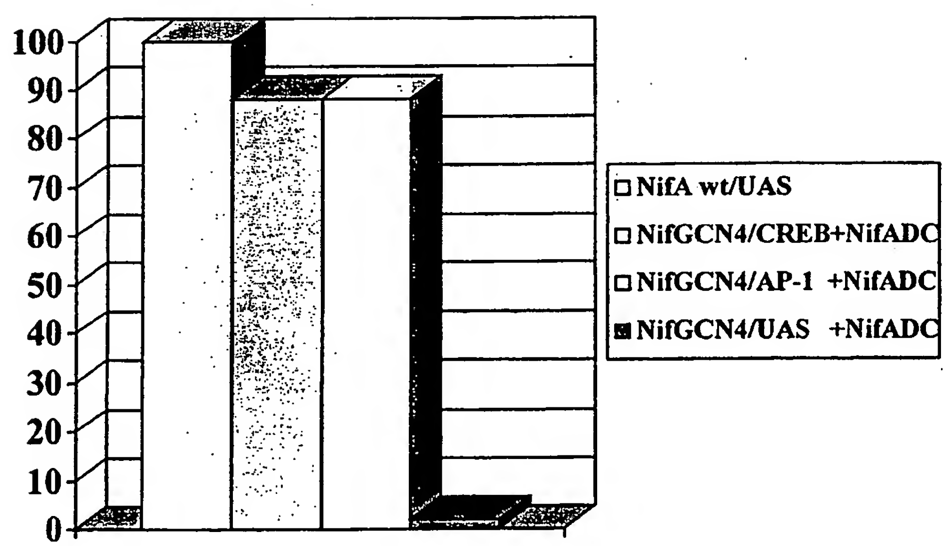

100
90
80
70
60
50
40
30
20
10
0
NifA wt/UAS
NifGCN4/CREB+NifADC
NifGCN4/AP-1 +NifADC
NifGCN4/UAS +NifADC

Figure 4

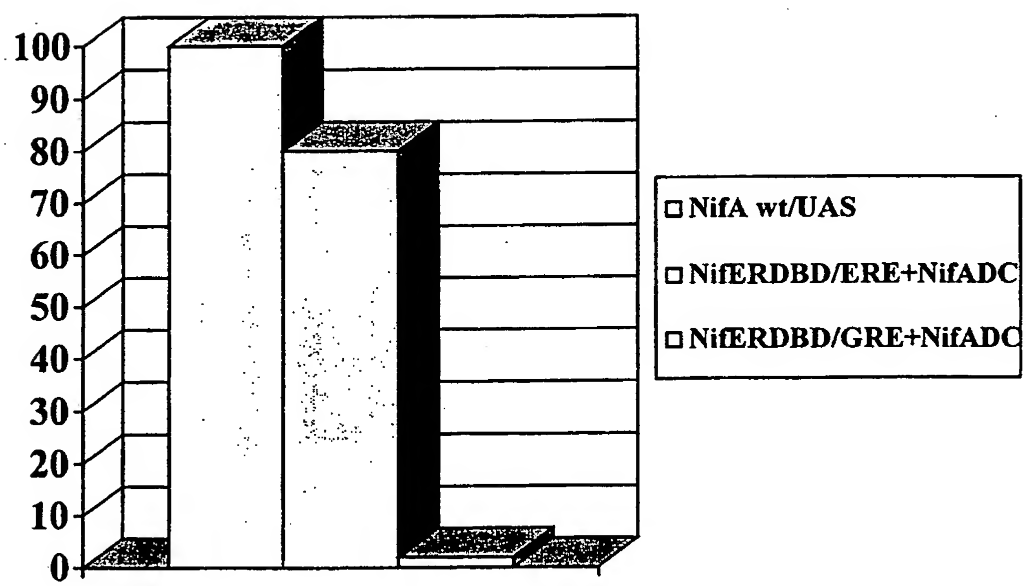
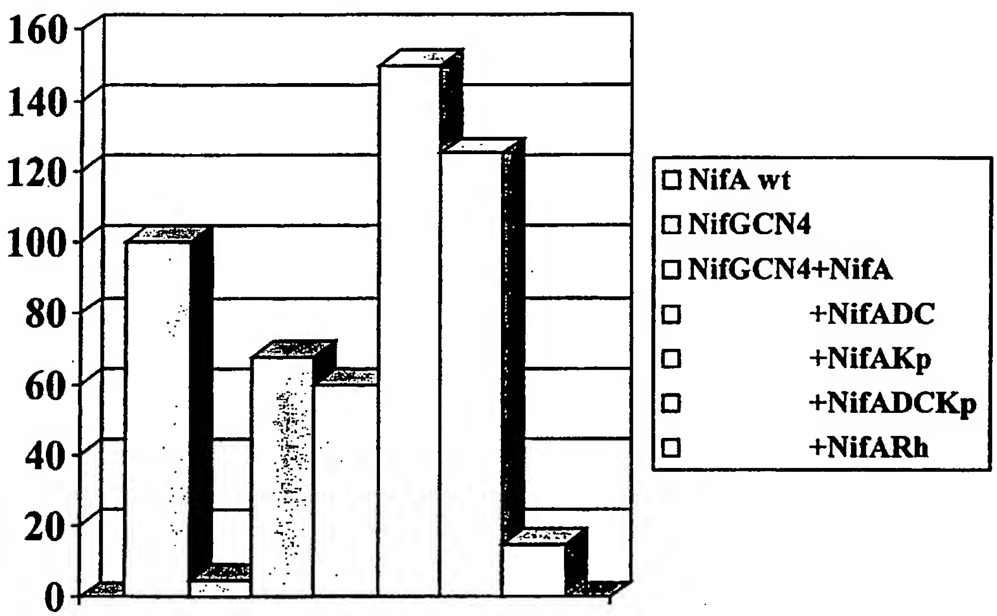

100
90
80
70
60
50
40
30
20
10
0
NifA wt/UAS
NifERDBD/ERE+NifADC
NifERDBD/GRE+NifADC

Figure 5


160
140
120
100
80
60
40
20
0
NifA wt
NifGCN4
NifGCN4+NifA
+NifADC
+NifAKp
+NifADCKp
+NifARh

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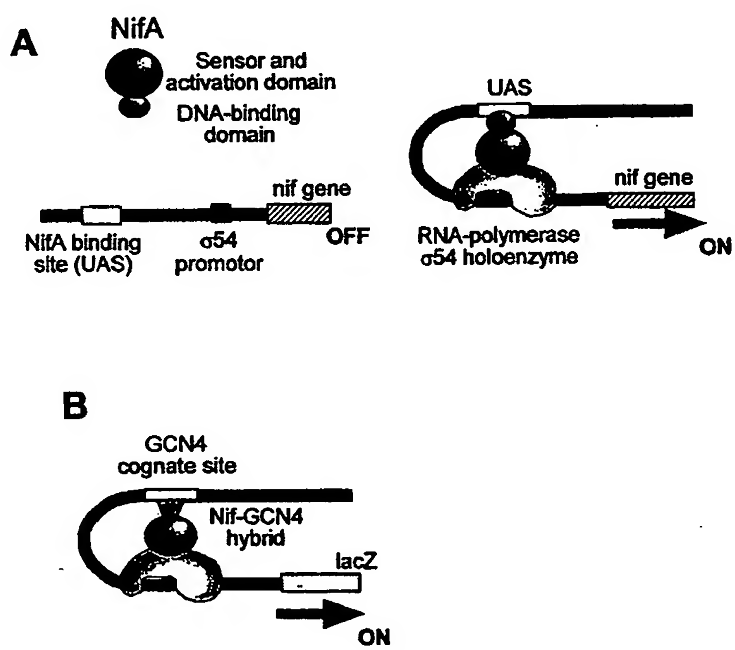
— *with international search report*

[Continued on next page]

(54) Title: SELECTION SYSTEM



(57) Abstract: A genetic selection system is described based on hybrid σ54 RNAP activator proteins. The invention permits the detection and screening for protein:DNA and protein:protein binding events.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/03450

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 G01N33/50 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data, MEDLINE, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 25947 A (SQUIBB BRISTOL MYERS CO) 18 June 1998 (1998-06-18) claims 1-24	1-27
A	WO 99 28746 A (LADANT DANIEL ;ULLMANN AGNES (FR); KARIMOVA GOUZEL (FR); PASTEUR I) 10 June 1999 (1999-06-10) claim 1	1-27

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 July 2001	11/07/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gabriels, J

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/03450

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9825947 A	18-06-1998	AU 721549 B	06-07-2000
		AU 5599398 A	03-07-1998
		EP 0963376 A	15-12-1999
		US 6051381 A	18-04-2000
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